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14. ABSTRACT
Radiotherapy (RT) is an important primary treatment for low-risk prostate cancer and the standard treatment for high-risk prostate cancer when combined with hormone therapy. Despite that many patients can be cured by RT, several studies suggest that approximately 30-60% of patients with high-risk cancer experience biochemical recurrence within five years after RT, among them 20% of patients die in 10 years.

Neuroendocrine differentiation (NED) is a process by which prostate cancer cells transdifferentiate into neuroendocrine-like (NE-like) cells, and NED is associated with disease progression and treatment failure. Based on our finding that the transcription factor cAMP response element (CREB) is responsible for fractionated ionizing radiation (FIR)-induced NED, we hypothesized that targeting neuroendocrine differentiation can sensitize prostate cancer cells to radiation. During the period of this grant support, we have made the following important discoveries. **First**, we have demonstrated that FIR-induced NED constitutes two distinct phases: acquisition of radiation resistance during the first two weeks and NED during the second two weeks. Further, we have demonstrated that targeting either phase can sensitize prostate cancer cells to radiation, and targeting both phases is a potent radiation sensitization approach. **Second**, we have identified PRMT5 as a critical upstream regulator of FIR-induced CREB activation and NED. Similarly, targeting PRMT5 during either of the two phases can sensitize prostate cancer cells to radiation. **Third**, we have discovered that PRMT5 is a novel epigenetic activator of AR transcription in prostate cancer cells and PRMTT5 expression correlates positively with AR expression in prostate cancer tissues. **Fourth**, we have generated preliminary data showing that PRMT5 may act as a master epigenetic regulator of IR-induced DNA DSBs. **Fifth**, we have also discovered that PRMT5 expression is transcriptionally regulated by NF-Y and post-translationally regulated by CHIP in prostate cancer cells. We have published 4 research articles and one review article and given 11 invited talks. Further, preliminary results generated through this support have allowed us to receive a 2015 Idea Development Award from PCRP and one RO1 from NIN/NCI. In addition, 32 students have conducted research in the lab through their thesis research, rotations and undergraduate research.

15. SUBJECT TERMS
Prostate cancer, LNCaP, DU-145, PC-3, neuroendocrine differentiation, NED
PRMT5, CREB, PKA, CaMKII, fractionated ionizing radiation, FIR, AR,CHIP, NF-Y

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1. Introduction

Prostate cancer remains the number one non-cutaneous cancer diagnosed and the leading cause of cancer deaths among American men. In 2017, 161,360 new patients will be diagnosed and 26,730 death will be reported [1]. Radiotherapy (RT) is a first-line treatment for low-risk prostate cancer and, when combined with neoadjuvant hormonal therapy, is a standard treatment for high-risk prostate cancer (PSA >20 ng/mL and/or clinical stage cT ≥ 3 and/or biopsy Gleason score ≥8) [2-4]. Importantly, RT is the most common treatment for patients who are 65-74 years old when compared with surgery and active surveillance [5]. Although a majority of prostate cancer patients are cured by RT, approximately 10% of patients with low-risk cancer and up to 30-60% of patients with high-risk cancer experienced biochemical recurrence within five years after RT and 20-30% of those relapsed died within 10 years [6-9]. Given that 96% of US patients presented with localized cancer, including 25% of patients with high-risk cancer [1, 10], failure to control these localized high-risk prostate cancers eventually leads to disease progression and contributes to the majority of prostate cancer deaths. Because RT and surgery are the only curative treatments for prostate cancer, enhancing the efficacy of prostate cancer cells to RT will have an enormous impact on reducing prostate cancer mortality.

Neuroendocrine (NE) cells represent a minor portion (<1%) of the epithelial cells in normal human prostate. Interestingly, NE-like cells, which also express NE markers such as chromogranin A (CgA) and neuron specific enolase (NSE), are present in almost all cases of prostatic adenocarcinoma and an increase in the number of NE-like cells is implicated in prostate cancer progression and is an indicator of poor prognosis [11-15]. A number of agents can induce prostate cancer cells to transdifferentiate intro NE-like cells, a process known as neuroendocrine differentiation (NED), via multiple pathways [16-27]. Because NE-like cells produce peptide hormones and growth factors that facilitate the growth of surrounding tumor cells in a paracrine manner and because NE-like cells are highly resistant to apoptosis [27-29], many studies have focused on establishing a clinical correlation between the extent of pre-existing NE-like cells and the therapeutic responses to RT and hormonal therapy and disease progression [11, 12, 25, 30, 31]. Because NED is reversible [32], these cells may be a dormant population under conditions of cellular stress and contribute to prostate cancer recurrence [15, 32]. The fact that hormonal therapy induces NED [33-38] suggests that therapy-induced NED may represent a novel pathway by which cancer cells survive treatment and contribute to tumor recurrence. This hypothesis is further supported by our recent findings that fractionated ionizing radiation (FIR) treatment also induces NED *in vitro*, *in vivo* and in prostate cancer patients [39, 40]. Based on the findings in literature and the preliminary studies, it was hypothesized that targeting the CREB signaling would inhibit RT-induced NED and enhance RT-induced cell killing. To test this hypothesis, three specific aims were proposed. **Aim 1** was to determine that targeting CREB can inhibit radiation-induced NED and increase radiation-induced cell killing *in vitro*. **Aim 2** was to determine that targeting critical upstream regulators of CREB can inhibit radiation-induced NED and increase radiation-induced cell killing *in vitro*. And **Aim 3** was to determine that targeting CREB signaling can inhibit radiation-induced NED and increase radiation-induced tumor killing *in vivo*. Under the support of this award, we have made the following progress during the grant period (September 30, 2013 – September 29, 2017).

2. Keywords

Prostate cancer, neuroendocrine differentiation, LNCaP, DU-145, PC-3, PRMT5, CREB, fractionated ionizing radiation, FIR

3. Overall Project Summary

Task 1. Aim 1: To determine that CREB targeting can inhibit radiation-induced NED and increase radiation-induced cell killing *in vitro* (months 1-18) **Completed!**

1a. Establish tetracycline-inducible stable cell lines using LNCaP, DU-145 and PC-3 cells.

Establishment of stable cell lines for CREB targeting was the major reagent we need to generate for the proposed work. We made two different types of shRNA expressing plasmids using the pRNATinH1.2 (Genescript) and pLKO-Tet-On (Addgene). The former relies on the availability of a stable cell line expressing Tet repressor whereas the later has the repressor encoding sequence in the same vector. We used pRNATinH1.2 to generate some shRNA constructs before. However, we recently switched to pLKO-Tet-On because of convenience to make stable cell lines with one transfection. We selected four validated targeting sequences from the Sigma Aldrich and used the last three digits corresponding to the Sigma TRCN sequence number (TRCN0000007**308**, TRCN0000226**467**, TRCN0000226**468**, and TRCn0000226**469**). We generated lentiviruses using these shRNA expressing plasmids and transduced the viruses into LNCaP cells for selection of cells that had stable integration of the plasmids. Western blotting analysis confirmed that induction of #468 shRNA by doxycycline (Dox+) showed 85% down-regulation of CREB when compared with non-induced control (Dox-), and that #467 and #469

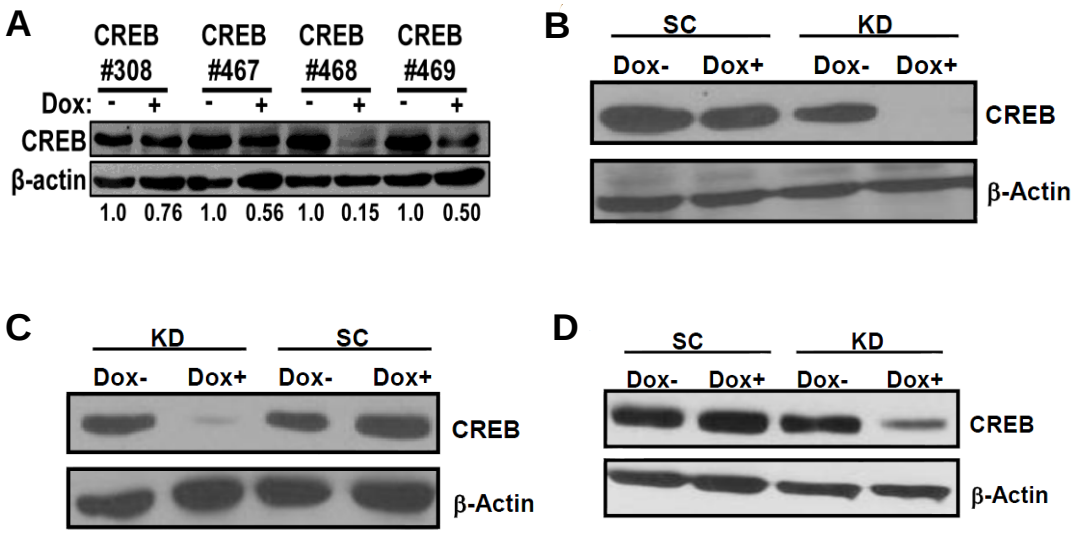

Figure 1. Establishment of prostate cancer stable cell lines expressing CREB shRNAs. A. Screening of CREB targeting sequences for establishment of CREB knockdown stable and doxycycline-inducible cell lines. Lentirviruses were generated for each of the shRNA plasmids and transduced into LNCaP cells for selection of stable integration of the plasmids for one week. Cells were induced with doxycycline (Dox+) at 1 μg/ml or without the induction (Dox-) for three days and harvested for Western blotting analysis of CREB expression. The numbers below the blot show the relative expression level when compared with Dox- for each stable cell line. **B-D**. Knockdown efficiency of CREB in established stable cell lines using LNCaP (B), DU-145 (C), and PC-3 (D). The #468 lentiviruses were used to establish independent stable cell lines using LNCaP, DU-145 and PC-3 cells, and efficient knockdown of CREB (KD) was observed when compared with Dox- or the scrambled control (SC).

showed approximately 50% down-regulation of CREB (Fig. 1A). We then used #468 lentiviruses to establish stable cell lines in LNCaP (Fig. 1B), DU-145 (Fig. 1C) and PC-3 (Fig. 1D). Induction of shRNA expression by doxycycline resulted in efficient knockdown of CREB in all three stable cell lines.

We previously used pcDNA4-TO system (Invitrogen) to establish stable cell lines expressing the dominant negative ACREB and observed that ACREB expression increased fractionated ionizing radiation (FIR)-induced cell killing (Fig. 4 in the proposal). However, we observed that non-induced cells also died when we performed long-term FIR. This is likely due to radiation-induced damage to the Tet repressor binding element in the promoter region. To circumvent this problem, we switched to the pLVX expression system (Clontech) that does not rely on the dissociation of the Tet repressor protein form the tetracycline-resistant operon, and established three stable cell lines using LNCaP cells. Doxycycline induction resulted in similar level of ACREB expression (Fig. 2). As CREB transcription is auto-regulated, it is evident that the expression level of CREB was also down-regulated, indicating that ACREB does act as a dominant negative mutant. We also tried to establish stable cell lines expressing ACREB in DU-145 and PC-3 cells. Unfortunately, we were unable to obtain any stable clones after three tries for the reason unknown. We decided not to pursue this as LNCaP is the best cell line that can be induced to undergo NED. These stable cell lines have been used in our 2014 ***Am J Cancer Res publication*** [41], which is included as an appendix in this report.

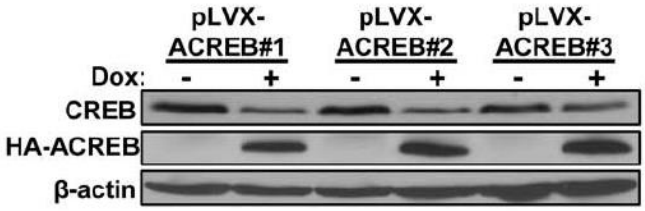

Figure 2. Establish of LNCaP stable cell lines that can inducibly express ACREB. The pLVX-Tet-On system from Clontech was used to establish three stable cell lines using LNCaP to inducibly express HA-ACREB by doxycycline (Dox). All three clones showed similar level of HA-ACREB induction and the down-regulation of CREB.

1b. Perform radiation-induced cell killing experiments using the established cell lines.

Using the established cell lines (#468) in Figure 1, we examined the effect of CREB knockdown on radiation-induced cell death. However, induction of CREB shRNAs during the first week did not increase FIR-induced cell death (Fig. 3). Similar results were observed when cells were irradiated for two weeks (20 Gy of FIR). The inability of CREB knockdown to increase FIR-induced cell death is not due to the selection of established stable clones as transient expression of CREB shRNAs also failed to increase FIR-induced cell death after 10 Gy of FIR, and another CREB knockdown construct targeting a different region of the CREB coding sequence yielded similar results. This is surprising, given that CREB phosphorylation was induced even after 10 Gy of FIR [40]. Because there are at least 3 members in the CREB/CREM/ATF-1 family that can form dimers with CREB to regulate target gene transcription [42], we reasoned that these family members might compensate for the reduction of CREB to regulate expression of target genes essential for cell survival. Alternatively, the residual amount of CREB might be sufficient to regulate expression of these target genes. Therefore, we performed similar experiments with ACREB stable cell lines. Because ACREB retains the ability to dimerize with endogenous CREB and other CREB dimerization partners but cannot bind

DNA, overexpressed ACREB can efficiently inhibit transcription of CREB target genes [43, 44]. We expected to see a potent effect with ACREB expression.

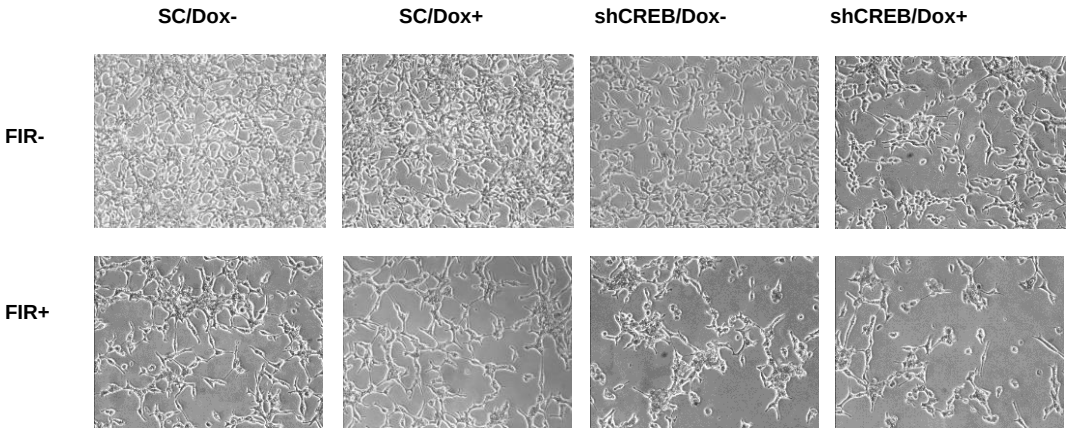

Figure 3. Effect of CREB knockdown on radiation-induced cell death. The established CREB knockdown cell line #468 (shCREB) and the stable cell line expressing scrambled control (SC) were induced with doxycycline (Dox+) at 1 μg/ml or without induction (Dox-) for two days, and then subjected 10 Gy of fractionated ionizing radiation (FIR+) or without irradiation (FIR-). Shown are representative images acquired 24 hours after the last irradiation.

Using the pcDNA4-TO-ACREB plasmid, we established four stable cell lines. Induction by tetracycline (Tet) resulted in expression of HA-ACREB in all four cell lines with the highest induction in ACREB#1 (Fig. 4A). Consistent with this, CREB expression was also down-regulated by 90%. Similarly, CREB expression in ACREB#4 was also down-regulated by 90%. We then performed MTT assays to determine the effect of ACREB expression on FIR-induced cell killing using the ACREB#1 cell line. As shown in Figure 4B, ACREB expression significantly increased FIR-induced cell killing in a dose-dependent manner. Similar results were observed with the ACREB#4 line. However, we did not see any significant effect of ACREB expression in ACREB#2 and ACREB#3 cell lines. Given that the CREB level in these two cell lines was only down-

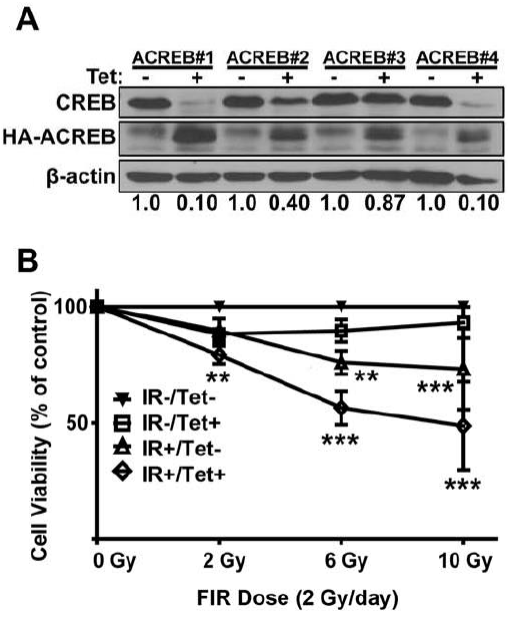

Figure 4. Effect of ACREB on radiation-induced cell death. **A**. Establishment of 4 independently isolated stable and tetracycline-inducible LNCaP clones expressing HA-ACREB using the pcDNA4TO expression system (Invitrogen). Induction of HA-ACREB inhibited auto-regulation of CREB. The numbers indicate relative level of tetracycline-induced (Tet+) CREB expression when compared with non-induced (Tet-). **B**. The stable cell line ACREB#1 in A was subjected to the indicated doses of fractionated ionizing radiation (FIR) (2 Gy/day) and cell viability was analyzed using the MTT assay. **, $P<0.01$; ***, $P<0.001$.

regulated by 60% and 13%, respectively, it is likely that efficient knockdown of CREB expression is necessary for FIR-induced cell killing.

To determine the effect of long-term expression of ACREB on FIR-induced cell death, we performed long-term FIR treatment. While attempting these experiments, using clones derived from the Invitrogen pcDNA6/TR/pcDNA4/TO expression system, there was excessive cell death under both induced and non-induced conditions, which is likely due to the effect of radiation-induced damage to the DNA encoding the tetracycline-resistance operon [45]. To overcome this problem, we utilized the Clontech pLVX-Tet-On lentiviral expression system that does not rely on the dissociation of the Tet repressor protein from the tetracycline-resistance operon [46]. Stable clones were prepared using three independent transductions and induction of ACREB sufficiently down-regulated the expression of CREB in each cell line (Fig. 2). To separate the role of CREB in both phases, we specifically induced ACREB expression during the NED phase only (weeks 3 and 4, post-20 Gy induction) and during the entire 4 weeks (pre-induction) to assess the impact of ACREB expression on the total number of viable cells at the end of 40 Gy FIR (Fig. 5A). Induction of ACREB during the entire FIR treatment period resulted in a 7.6-fold reduction in cell number, and induction of ACREB during the NED phase also resulted in a 2.5-fold reduction (Fig. 5B). Because of extensive cell death, we were unable to assess the impact of ACREB on chromogranin A (CgA) and neuron specific enolase (NSE) expression. However, some of the remaining survival cells only displayed short neurites. These results demonstrate that CREB plays a critical role in the acquisition of radioresistance and the acquisition of NED during the process of FIR-induced NED.

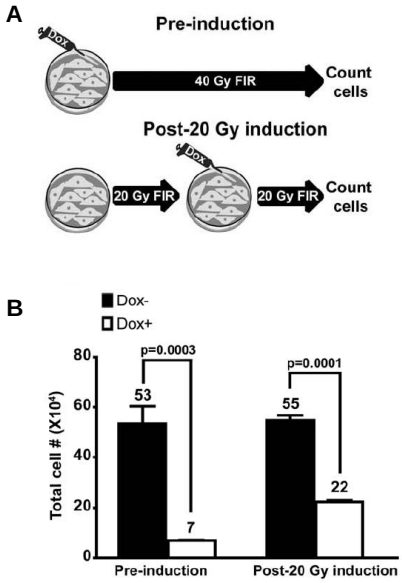

Figure 5. Effect of ACREB expression on radiation-induced cell death during the course of FIR-induced NED. A. Shown are two experimental designs to determine the effect of HA-ACREB expression on cell survival shown in B. HA-ACREB were induced by Dox during the entire 40 Gy of FIR (Pre-induction) or during NED acquisition phase only (Post-20 Gy induction). **B**. The established three stable cell lines in Figure 2 were subjected 40 Gy of FIR (2 Gy/day, 5 days/week), and doxycycline (Dox) at 1 μg/ml was added during the entire four weeks or during the last two weeks as designed in A. The number of viable cells was determined by Trypan Blue Exclusion at the end of 40 Gy irradiation, and Student's *t*-test was applied for statistical analysis.

1c. Perform radiosensitization experiments using the established cell lines (clonogenic assay) (months 9-15). To determine whether targeting CREB can radiosensitize prostate cancer cells, we performed clonogenic assays using the established LNCaP stable cell line expressing ACREB (Fig. 2). We observed significant radiosensitization in all doses when ACREB was expressed (Fig. 6A). Because clonogenic assay assesses the reproductive ability of cells after a single exposure, which

is different from FIR in which DNA damages could be repaired during the interval of irradiation by functional compensation of other CREB family members, we also performed clonogenic assay with the LNCaP stable cell line expressing CREB shRNA#468 (Fig. 1B). Indeed, knockdown of CREB also sensitized LNCaP cells to radiation in a dose-dependent manner (Fig. 6B). These results collectively suggest that targeting CREB can sensitize LNCaP cells to radiation.

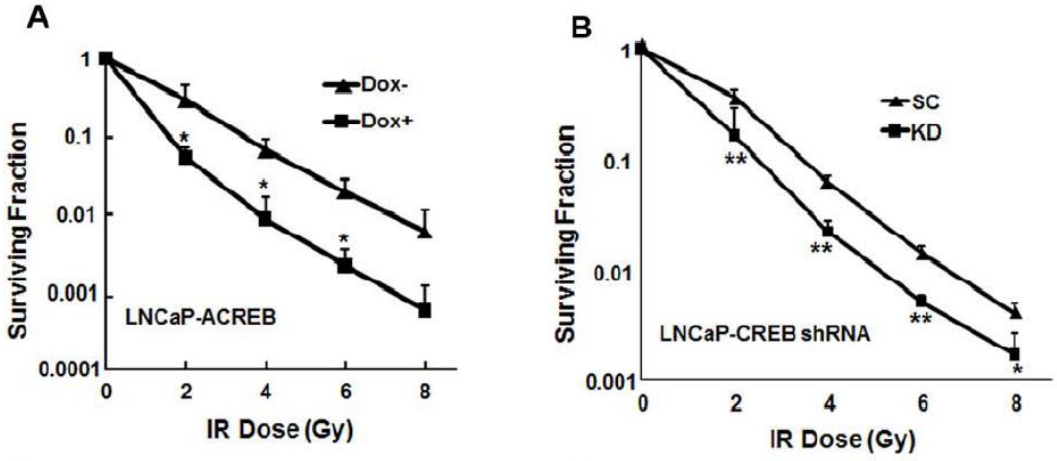

Figure 6. CREB targeting sensitizes prostate cancer cells to radiation. Indicated stable and doxycycline-inducible LNCaP cell lines expressing HA-ACREB (A) or CREB shRNA#468 (KD) (B) or scrambled control (SC) were induced to express HA-ACREB for 48 hours or CREB shRNA#468 for 72 hours and then subjected to a single exposure of the indicated dose of IR, followed by seeding of various numbers of cells in 6-well plates for colony formation. Colony formation was counted 2 weeks later and survival fraction was calculated. Shown are mean from three independent experiments. *, P <0.05; **, P <0.01.

To extend our finding to DU-145 and PC-3 stable cell lines, we used our established doxycycline-inducible stable cell lines and the scrambled control (SC) cell lines to perform colonogenic assays. As shown in Figure 5 in 2014 AJCR paper [41], knockdown of CREB sensitized DU-145 to radiation. However, knockdown of CREB did not sensitize PC-3 cells to radiation. This is consistent with inefficient induction of NED induced by FIR and slight activation of CREB in PC-3 cells as reported in 2011 AJCR [39]. These results were presented in these two papers and are submitted in this report as Appendix.

1d. Perform radiation-induced NED experiments (Months 15-24) To determine whether CREB targeting can increase IR-induced cell death by inhibiting IR-induced NED, we performed 40 Gy of FIR and evaluated the expression of NE markers CgA and NSE, and observed that knockdown of CREB almost completely inhibited the expression of NSE. Interestingly, CREB knockdown did not exhibit any effect on CgA expression. This is likely due to functional compensation by other CREB family members. Nevertheless, CREB knockdown significantly inhibited neurite outgrowth. These results are presented in Fig. 1B and 1C in the 2014 AJCR paper [41]. For details, please refer to the attached Appendix in this report.

Task 2. Aim 2: To determine that targeting critical upstream regulators of CREB can inhibit radiation-induced NED and increase radiation-induced cell killing *in vitro* (Months 13-36) **completed.**

2a. Time course of PKA/CaMKII activation by IR (Months 13-18).

As discussed below in 2c, we determined the role of PKA and CaMKII in IR-induced NED and cell death by performing 40 Gy of fractionated IR in the presence or absence of specific inhibitors for PKA and CaMKII. Although we observed almost complete inhibition of IR-induced CREB phosphorylation at Ser133, and/or partial suppression of CgA expression, we did not see any apparent effect on NED and cell death. These new observations confirmed our previous observation that CREB-S133A failed to inhibit IR-induced NSE and CgA expression. It is worth noting that CREB-S133A only suppressed the elongation of neurites without obvious effect on cell viability and the growth of short neurites. In contrast, targeting CREB by using knockdown or ACREB effectively inhibited IR-induced NED and sensitized prostate cancer cells to radiation as outlined in Task 1. Taken all of these observations together, we hypothesize that additional protein kinases might be involved by phosphorylating additional sites in CREB. Instead of measuring the kinase activity of PKA and CaMKII, we will try to use mass spectrometry to identify phosphorylation sites and possibly upstream protein kinases.

2b. Determine the involvement of PKA/CaMKII-CREB pathway in the DNA damage response (Months 13-24)

Since inhibition of PKA and CaMKII did not sensitize LNCaP cells to IR, it is likely that these two protein kinases are not involved in the acquisition of radioresistance. Because targeting CREB increased IR-induced cell death and sensitized prostate cancer cells to IR, we investigated the underlying mechanisms by analyzing the effect of CREB targeting on DNA damage response. We found that targeting CREB in LNCaP cells primarily increased IR-induced pre-mitotic apoptosis, and to a lesser extent post-mitotic apoptosis. The IR-induced apoptosis is due to increased activation of caspase-3. However, there is no obvious effect on cell cycle and autophagy. These results are presented as Fig. 3 and Fig. 4 in the 2014 AJCR paper [41]. For details, please refer to the attached Appendix.

2c. Determine the role of PKA/CaMKII in IR-induced NED (Months 25-36).

Since inhibition of PKA by H-89 significantly suppressed IR-induced CREB phosphorylation at Ser133 (pCREB), we performed long-term treatment during 40 Gy of fractionated IR and evaluated the role of PKA in IR-induced NED. As shown in Fig. 7, H-89 failed

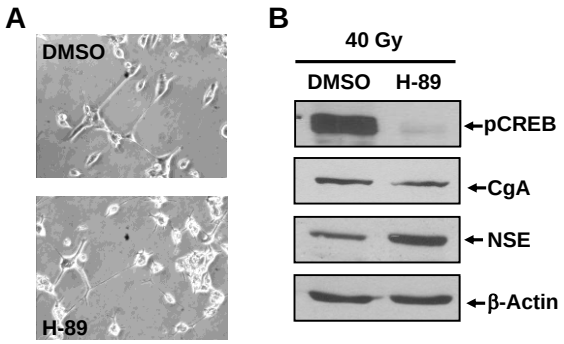

Figure 7. Effect of PKA inhibition on IR-induced NED and cell survival. LNCaP cells were subjected to 40 Gy of fractionated IR (2 Gy/day, 5 days/week) in the presence of the PKA inhibitor H-89 (10 μM) or the control DMSO. **A**. Representative images acquired at the end of 40 Gy irradiation. **B**. Western blotting analysis of CREB phosphorylation at Ser133 (pCREB), CgA and NSE. Cells were harvested at the end of irradiation treatment and total cell lysate was prepared and used for the detection of the indicated proteins by Western blotting.

to inhibit the outgrowth of neurites and similar number of cells survived when compared with the control, despite that IR-induced pCREB was almost completely suppressed. Interestingly, there was no obvious effect on CgA and NSE expression. This result suggests that PKA is unlikely involved in IR-induced NED.

To evaluate the effect of CaMKII on IR-induced NED, we performed similar fractionated IR treatment in LNCaP cells in the presence or absence of the CaMKII inhibitor KN-93. Interestingly, inhibition of CaMKII by KN-93 did not result in increased cell death though neurite outgrowth was partially inhibited. Instead, most survived cells were enlarged (Fig. 8A). Western blotting analysis revealed that pCREB and CgA expression was almost completely inhibited after 40 Gy of fractionated IR (Fig. 8B). However, there was no apparent effect on NSE expression. Given that targeting CREB by ACREB or CREB knockdown (Task 1) does inhibit IR-induced NED and sensitize cells to IR, it is possible that phosphorylation of S133 in CREB may not be responsible for IR-induced cell death. This is also consistent with our previous observation that a non-phosphorylatable mutant CREB-S133A failed to inhibit IR-induced CgA and NSE expression. Taken together, these new observations favor our hypothesis that additional protein kinases might be involved in IR-induced CREB activation via additional phosphorylation sites. To test our hypothesis, we will use mass spectrometry to discover additional phosphorylation that may be induced by IR in prostate cancer cells.

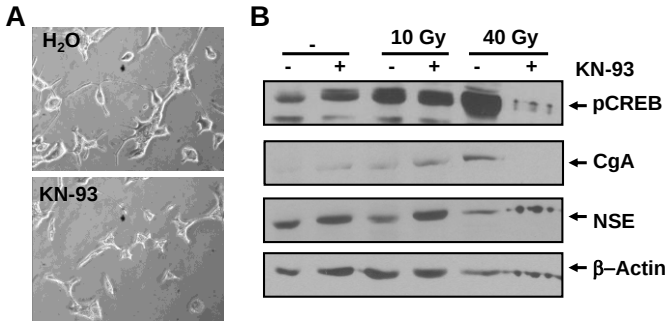

Figure 8. Effect of CaMKII inhibition on IR-induced NED and cell survival. LNCaP cells were subjected to 10 or 40 Gy of fractionated IR (2 Gy/day, 5 days/week) in the presence of the CaMKII inhibitor KN-93 (10 μM) or the control H_2O. **A**. Representative images acquired at the end of 40 Gy irradiation. **B**. Western blotting analysis of CREB phosphorylation at Ser133 (pCREB), CgA and NSE. Cells were harvested at the end of irradiation experiments and total cell lysate was prepared and used for the detection of the indicated proteins by Western blotting.

2d. Establish tetracycline-inducible stable cell lines to knock down PRMT5 (Months 13-18).

We successfully established stable cell lines to inducibly knock down PRMT5 using two different shRNA sequences. As shown in Fig. 9, induction of shRNA expression efficiently knocked down PRMT5.

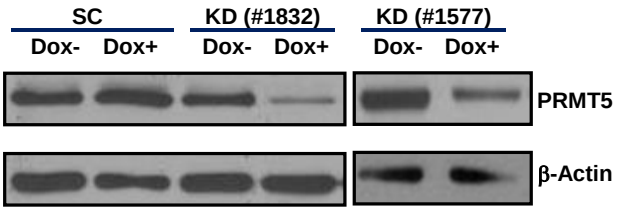

Figure 9. Establishment of LNCaP stable cell lines that inducibly knock down PRMT5. LNCaP cells were transfected with two shRNA constructs targeting two different regions (#1577 and #1832) or the scrambled control (SC), and stably integrated clones were individually isolated. Shown is the Western blotting detection of PRMT5 expression with the induction by doxycycline (Dox+, 1 μg/ml) for 96 hours, or without induction (Dox-).

2e. Effect of PRMT5 knockdown on IR-induced NED (Months 19-24).

We have found that knockdown of PRMT5 during first week dramatically increased IR-induced cell death (Fig. 10A). To assess whether PRMT5 knockdown sensitizes prostate cancer cells to IR, we performed clonogenic assays in LNCaP cells, and found that knockdown of PRMT5 significantly sensitized LNCaP cells to IR (Fig. 10B). To extend this exciting finding, we also performed clonogenic assays in DU-145 and PC-3 cells that do not express AR. Remarkably, knockdown of PRMT5 also sensitized these two cells to radiation (Fig. 10C and 10D).

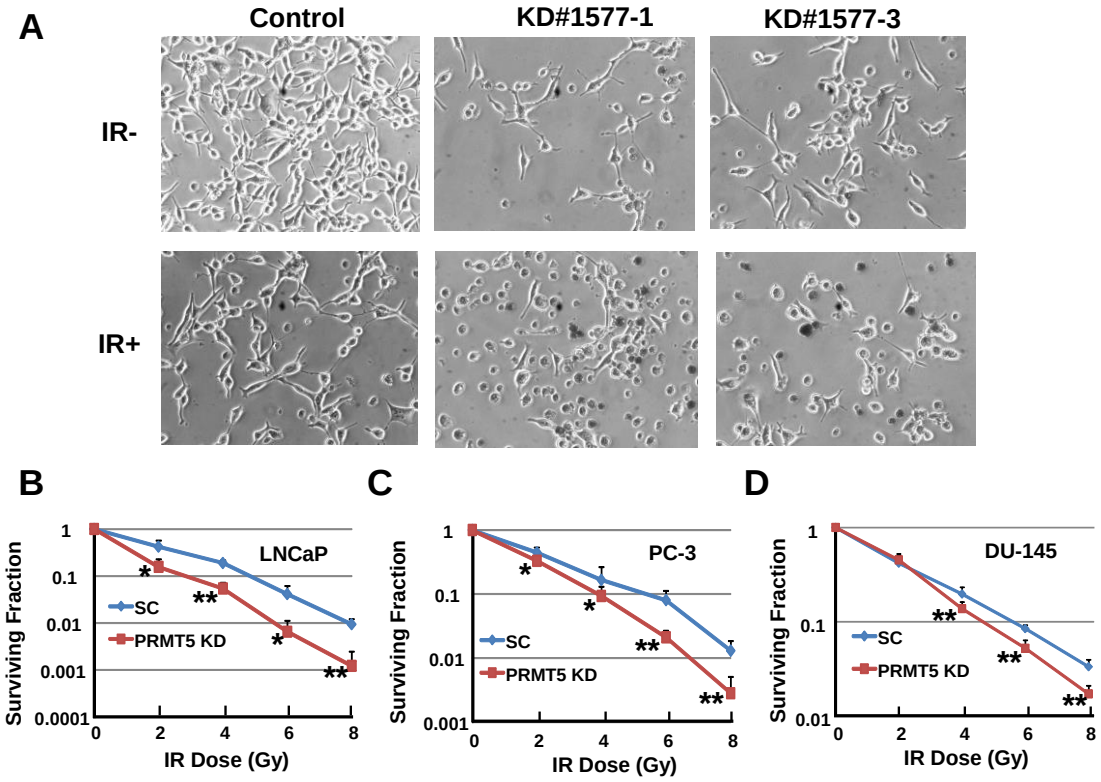

Figure 10. Knockdown of PRMT5 increases IR-induced cell death and radiosensitize prostate cancer cells. A. LNCaP cells were transfected with the PRMT5 shRNA#1577 (clone #1 and #3) or the control (Con) for 48 h, followed by IR (2 Gy/day) for three days (IR+). Similar control experiment was performed without irradiation (IR-). Phase contrast images shown were taken 24 h after the third irradiation. **B-D**. The indicated prostate cancer cells were transiently transfected with the PRMT5 shRNA#1577 for 48 h, and then subjected to the indicated dose of IR. Cells were immediately trypsinized and counted, and various numbers of cells were seeded in 6-well plates for the formation of colonies for 14 days. The number of colony was counted and surviving fraction was calculated. Results are presented as means from three independent experiments, and students *t*-test was used to determine statistical significance (*P<0.05; **P<0.01).

To determine whether PRMT5 knockdown can inhibit IR-induced NED, we used our established stable cell lines to induce PRMT5 knockdown during the second two weeks, which is the phase of NED induction. As shown in Fig. 11A, knockdown of PRMT5 expression by doxycycline dramatically increased IR-induced cell death when compared with non-induced or SC controls. When PRMT5 expression was knocked down during the entire 4 weeks of IR treatment (Fig. 11B), almost all cells died. Because few cells survived the treatment, it was impossible to

analyze the expression of NED markers CgA and NSE. However, it is obvious that few survived cells barely showed extended neurite outgrowth when compared with controls (Fig. 11). In conclusion, we believe that PRMT5 confers radioresistance in prostate cancer cells and mediates FIR-induced NED. These results also support the notion that targeting NED itself is sufficient to radiosensitize prostate cancer cells.

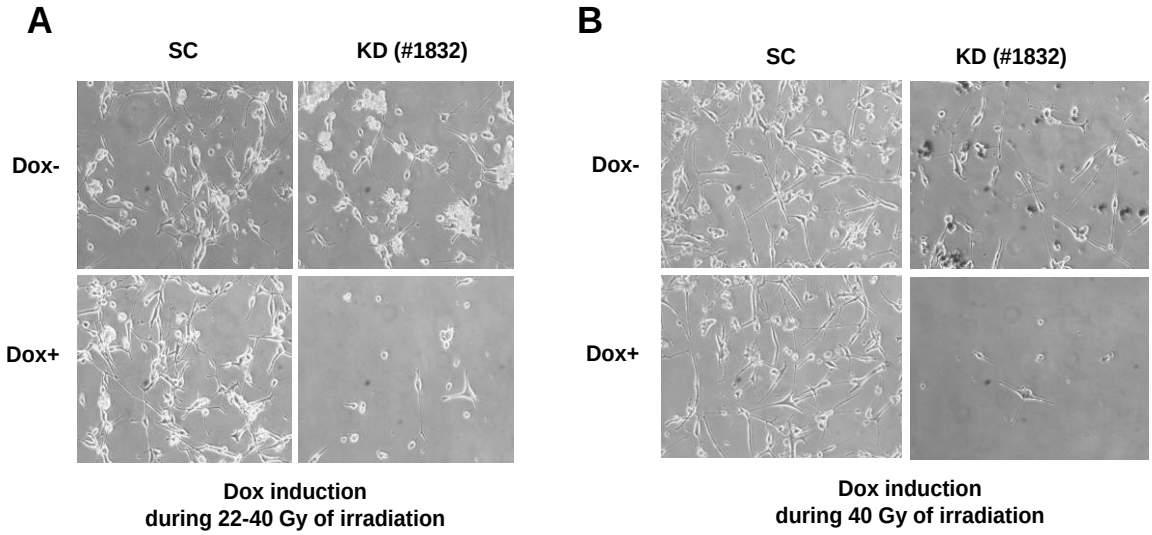

Figure 11. PRMT5 knockdown inhibits IR-induced NED in LNCaP cells. The established stable cell line that inducibly expressed PRMT5 shRNA#1832 or SC was used to evaluate the effect of PRMT5 knockdown by doxycycline (Dox+) on IR-induced NED. Because IR-induced NED constitutes two phases: acquisition of radioresistance during the first two weeks and acquisition of NED during the second two weeks, we specifically designed the experiments by inducing PRMT5 knockdown during the NED phase (**A**) and during the entire 4 weeks of irradiation (**B**). In either case, almost all cells died and IR-induced NED was dramatically suppressed when PRMT5 knockdown was induced.

2f. Elucidate the mechanism by which PRMT5 regulates IR-induced CREB activation (Months 25-36).

We hypothesized that PRMT5 may methylate CREB and mediate IR-induced activation. However, *in vitro* reconstitution assay using purified CREB and immunoprecipitated PRMT5 did not result in conclusive results (Fig. 12). Since we suspected that Western blotting-based method may not be sensitive enough, we are planning to use radioisotope-labeled SAM to perform similar experiments.

We also searched the CREB sequence and identified arginine residues R130 and R131 as potential methylation sites. We hypothesized that methylation of one of these arginine residues may impair or inhibit FIR-induced CREB phosphorylation at S133. As a matter of fact, a recent report also provided evidence that a CREB peptide containing R130 and R131 can be methylated by PRMT5 [47].

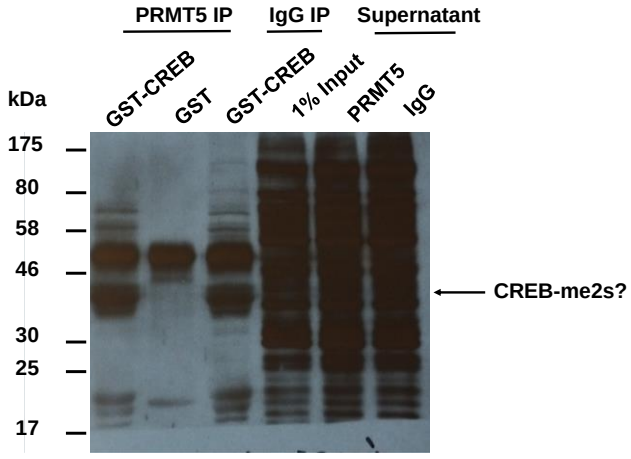

Figure 12. *In vitro* methylation of CREB by PRMT5. LNCaP cells were cultured to 80% confluency and total lysate was prepared for immunoprecipitation with either anti-PRMT5 antibody (PRMT5 IP) or the control IgG (IgG IP). The 1% of total lysate used for IP was loaded as input, and supernatant after IP was also loaded for both PRMT5 IP and IgG IP. Bacterially expressed and purified GST-CREB or GST was added into the methylation reaction at 5 μg in the presence of 100 nmol of SAM in a total reaction of 30 μl. The reaction was incubated at 30°C for 30 min and was stopped by adding 6 μl of 6x Laemmli loading buffer. The sample was boiled at 30°C for 5 min and loaded into 10% SDS-PAGE for electrophoresis, followed by Western blotting analysis with anti-mono and dimethyla arginine antibody (Abcam, 7E6, ab412). CREB-me2s, dimethylated arginine residues in CREB.

To test our hypothesis and to confirm that IR does induce PRMT5-mediated methylation of R130 and R131 at the endogenous level, we are currently generating methylation-specific antibodies using a peptide containing methylated R130 and R131. Once we identify such antibodies, we will be able to evaluate the impact of IR-induced CREB methylation in FIR-induced NED. This is particularly important as mass spectrometry did not allow us to identify methylated peptides from CREB.

Task 3. Aim 3: To determine that targeting CREB signaling can inhibit radiation-induced NED and increase radiation-induced tumor killing *in vivo* (Months 7-30). **Ongoing**.

3a. Submit animal protocols for approval from Purdue University and USAMRMC (Months 1-6).

We submitted our initial protocol and received approval on April 18, 2013. Due to the expiration of our previous animal protocol at Purdue University, we resubmitted a new protocol in 2015, which was approved by USAMRMC on May 1, 2015.

3b. Optimize tetracycline concentrations for induction of ACREB and CREB shRNAs in xenograft tumors (Months 7-12).

Because either CREB knockdown or ACREB failed to completely suppress the function of CREB in cells, targeting CREB by knockdown or ACREB may not faithfully reflect the function of CREB *in vivo*. As we identified PRMT5 as a critical regulator to mediate FIR-induced CREB activation and NED, we decided to perform *in vivo* targeting experiments with PRMT5 knockdown. To optimize the doxycycline induction condition in xenograft models, we established xenograft tumors in male athymic nude mice (5-7 week old) using LNCaP-shPRMT5 and LNCaP-SC. Mice were fed drinking water containing Dox at 1 mg/ml and PRMT5 expression was knocked down as shown in Fig. 5c and 5d in our 2017 Oncogene paper [48]. Significantly, knockdown of PRMT5 completely suppressed the growth of xenograft tumors in mice. These results are provided in the Appendix in this report.

3c. Perform CREB targeting on IR-induced NED in mice (Months 13-18).

We acquired an X-ray (X-RAD 320) from Precision with the generous support from Purdue University Vice President for Research Office, College of Pharmacy, and Purdue University Center for Cancer Research. Due to a delayed move to the new lab and a repairing of the X-RAD 320 irradiator, we performed the first radiation experiment in 2016 (9/28/2016-12/8/2016). We implanted LNCaP-shPRMT5 cells into 26 mice and then monitored tumor growth. We began doxycycline (Dox) treatment (1mg/ml in drinking water) when average tumor volume reached to 200-300 mm^3, mice were divided into two groups (Dox+ and Dox-) and performed 5 fractions of IR (2 Gy/fraction, one fraction/day). Mice were fed normal drinking water after the completion of radiation treatment and tumor growth was monitored. Tumors regressed during FIR treatment in both control (Dox-) and treated (Dox+) group, but began to regrow after the completion of FIR. Although tumors began to regrow immediately after the completion of FIR treatment in Dox- group, tumors in treated group stayed flat for almost 20 days. However, tumor growth resumed in both control and knockdown groups after one month observation (day 47-78), though the average tumor volume in knockdown group is slightly lower than untreated group (Fig. 13). This resutl suggests that 5 fractions of IR may not be sufficient to induce sustained tumor killing *in vivo*.

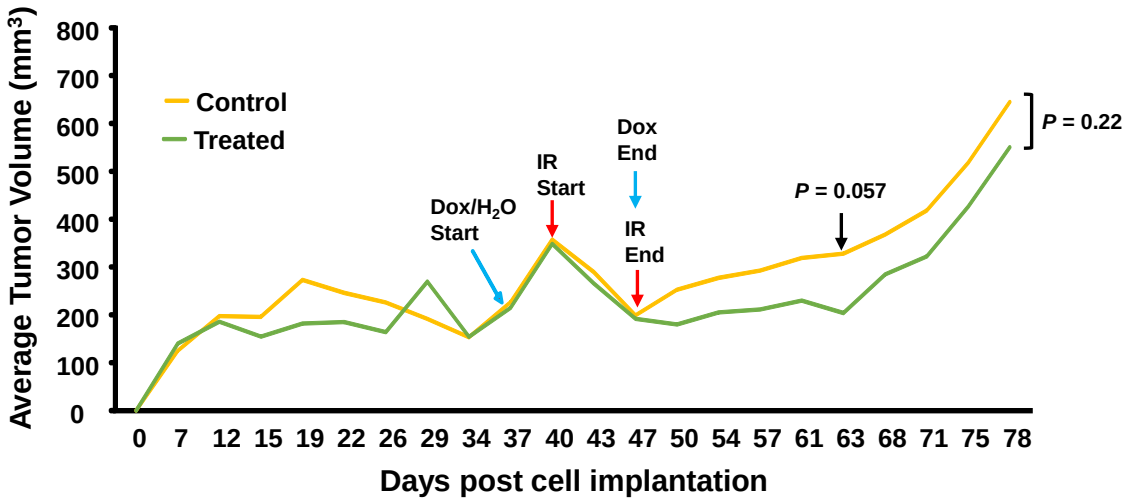

Figure 13. Effect of PRMT5 knockdown on LNCaP xenograft tumor growth. The doxycycline (Dox)-inducible PRMT5 knockdown cell line LNCaP-shPRMT5 was injected into 26 male athymic nude mice ($3x10^6$ cells) (ages 6-8 weeks) and tumor growth was monitored. Knockdown of PRMT5 was induced by Dox (1mg/ml) in drinking water when tumor volumes reached to 200-300 mm^3 and then subjected to fractions of IR treatment (2 Gy/fraction). Dox treatment was terminated after the completion of IR treatment and tumor growth was monitored for one month. Student's *t*-test was used to determine the statistical difference in average tumors between treated and control groups at the last day observation and at days 63 after implantation.

3d. Perform CREB targeting on tumor regrowth (Months 19-30).

To determine whether targeting FIR-induced NED can reduce tumor recurrence rate, we implanted both LNCaP-SC and LNCaP-shPRMT5 into 40 immunocompromised mice. When tumor volume reached to 200-300 mm^3, mice treated with doxycycline-containing drinking water (1mg/ml) to induce expression of SC or PRMT5 shRNAs and subjected to a total of 40 FIR (5 Gy/day, twice/week). After the completion of FIR, mice were fed with normal drinking water

without doxycycline. Tumor growth were observed twice a week. Tumor implantation was performed on October 6, 2017, and we are currently performing 40 Gy of FIR. The last radiation will be completed by December 29, 2017. We will observe tumor regrowth in both SC and knockdown groups for 3-6 months depending on tumor recurrence rate. We anticipate this experiment will be completed at least by June, 2018.

Additional accomplishments relevant to proposed research

Transcriptional regulation of PRMT5 expression in prostate cancer. Since we observed that PRMT5 expression is induced by FIR, we investigated how PRMT5 expression is transcriptionally regulated. We systematically characterized the promoter of PRMT5 in prostate cancer cells and identified NF-Y as the major transcriptional activator of PRMT5 expression. Furthermore, we also found that activation of the PKC signaling down-regulates NF-Y and inhibits PRMT5 expression in a c-Fos-dependent manner. This finding suggests that the PKC/c-Fos signaling is a negative regulator of PRMT5 expression. Consistent with this, we also found that the expression level of several PKC isozymes inversely correlates with PRMT5 expression in prostate cancer. Our finding is also in support of recent finding that PKC may actually function as a tumor suppressor rather than an oncoprotein [49]. Our finding represents the first study of transcriptional regulation of PRMT5 expression. This work has been published in Biochimica et Biophysica Acta (Zhang et al., 1839:1330-1340, 2014), and is included in this report as Appendix.

Radiation-induced NED as a mechanism of radioresistance in prostate cancer and treatment failure. I was invited by Dr. Mercedes Salido, who edited a topic issue on neuroendocrine differentiation in prostate cancer for publication in Frontier Oncology, to write a review on radiation-induced NED in prostate cancer for publication in a special topic issue of "Neuronendocrine differentiation in Prostate Cancer: new insights and clinical implications." In collaboration with Dr. Richard Choo at Mayo Clinic and Dr. Jiaoti Huang at UCLA, we wrote and published a review titled "neuroendocrine differentiation in prostate cancer: A mechanism of radioresistance and treatment failure." We propose a model by which radiation induces NED and suggest possible targeting strategies in the review. This review article was published on April 14, 2015, and is also included in this report as Appendix.

PRMT5 regulates prostate cancer cell growth via epigenetic activation of AR transcriptio*n.* During the course of evaluating the effect of PRMT5 knockdown on radiation-induced cell killing in LNCaP cells, we observed that PRMT5 knockdown by itself also inhibited cell growth (Fig. 10). We then tested its effect on cell growth in DU-145 and PC-3 cells and observed the lack of effect. This observation suggested that regulation of prostate cancer cell growth by PRMT5 may be dependent on the AR status. Because AR is the critical driver of prostate cancer development and progression and is the therapeutic target of ADT, we believe investigating the role of PRMT5 in regulation of AR expression may provide evidence that PRMT5 is not only a therapeutic target for prostate cancer radiosensitization but also is a therapeutic target for development of novel treatment for castration resistant prostate cancer (CRPC). Thus, we invested our effort on this and confirmed that PRMT5 acts as a novel epigenetic activator of AR transcription in prostate cancer cells, and this work was recently published in Oncogene (2017), which is included in this report as appendix. The major findings reported in the Oncogene paper are as follow [48]:

(1) PRMT5 regulates prostate cancer cell growth in an AR-dependent manner.
(2) PRMT5 binds to the AR promoter and epigenetically regulates AR transcription.
(3) PRMT5 is recruited to the AR proximal promoter region by its interaction with Sp1, and Brg1, an ATP dependent chromatin remodeler, is involved in epigenetic regulation of AR transcription.
(4) PRMT5 is overexpressed in prostate cancer tissues, and its expression correlates positively with AR expression.
(5) PRMT5 knockdown completely suppressed the growth of LNCaP xenograft tumors in mice.

PRMT5 is required for the repair of DNA double-strand breaks. The effect of radiotherapy is largely determined by the induction of DNA double-strand breaks (DSBs). Because our preliminary observations show that FIR dose-dependently induces PRMT5 expression and because PRMT5 was also reported to regulate the function of p53 and Rad9, both of which are involved the repair of DNA damages [50, 51], we examined whether PRMT5 regulates the repair of DSBs. We transfected LNCaP cells with PRMT5 shRNA or the SC control for three days and then subjected cells to different doses of radiation. As γH2AX is a hallmark of DSBs, we performed immunocytochemical staining and Western blotting analysis of γH2AX (Fig. 14A and 14B). Indeed, a significant induction of γH2AX foci formation and expression level was observed. Consistent with this, Comet Assay also indicates the increased induction of DSB when compared with SC (Fig. 14). These results together suggest that PRMT5 is required for the repair of radiation-induced DSBs.

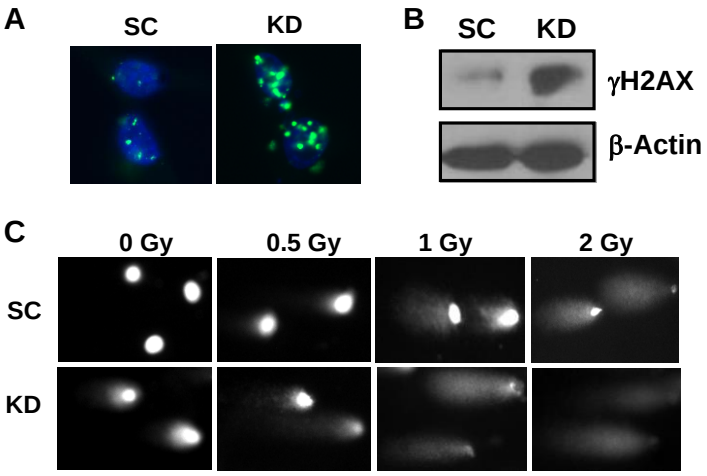

Figure 14. Knockdown of PRMT5 increases IR-induced DNA double-strand break. A. LNCaP cells transiently transfected with scrambled control (SC) or PRMT5 shRNA (KD) plasmids for 48 h were irradiated with 1 Gy, and γH2AX staining was performed 2 h after the irradiation. **B**. Similar treatment was performed as in A and total cell lysate was prepared for immunoblotting analysis of γH2AX. **C**. LNCaP cells were transfected with SC or PRMT5 shRNA plasmids for 48 h, followed by irradiation of the indicated dose for Comet assay.

PRMT5 epigenetically regulates expression of genes required for DSB repair. Although it is known that PRMT5 regulates DNA damage by post-translational modification of p53, our results showed that knockdown of PRMT5 also sensitized PC-3 cells, which is deficient in p53, to IR. In addition, the posttranslational modification of Rad9 by PRMT5 is not involved in the repair of DSBs induced by IR [50]. As PRMT5 is an epigenetic regulator, we reasoned that PRMT5 may regulate IR-induced DSBs repair by epigenetic regulation of target genes involved in DSBs repair. To this end, we performed RNA-seq analysis in the presence or absence of PRMT5 knockdown to identify target genes in response to IR treatment. As shown in Fig. 15A, we have identified 121 genes that are differentially expressed. Interestingly, 85% (103) of genes are down-regulated when PRMT5 is knocked down, suggesting that PRMT5 mainly activates transcription of these target genes in LNCaP cells. Note that the majority of identified genes are involved in DNA damage response. To confirm several genes that are involved in DNA damage response, we further performed qPCR and confirmed that RAD51AP1, RAD51D, RAD51 and NHEJ1 are down-regulated by PRMT5 knockdown (Fig. 15B). These results suggest that PRMT5 may contribute to both homologous recombination (HR) and NHEJ by epigenetically activating transcription of these target genes. We are currently characterizing PRMT5's role in regulation of HR and NHEJ.

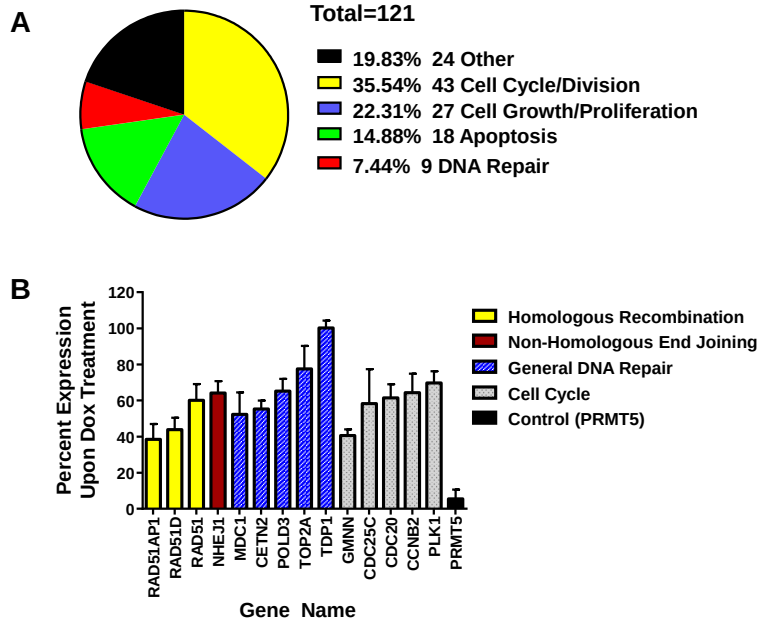

Figure 15. PRMT5 regulates many genes involved in the DNA damage response. **A**. Identification of 121 genes differentially expressed upon PRMT5 knockdown induced by doxycycline. LNCaP-shPRMT5 cells were incubated in the presence or absence of Doxycycline (Dox) for three days followed 2 Gy of ionizing radiation. RNA was isolated 24 h after the radiation treatment. Three independent experiments were performed and the total RNA samples were subjected to Illumina next-generation sequencing in Purdue Genome Core facility, and the data was analyzed by the Purdue University Bioinformatics Core facility. **B**. Percent expression of selected genes including four involved in HR and NHEJ that are down-regulated upon doxycycline (Dox) treatment to induce PRMT5 knockdown when compared with Dox- (100%).

4. Key Research Accomplishments

- We have successfully demonstrated that CREB is a critical transcription factor mediating FIR-induced NED. Using various approaches, we have also demonstrated that targeting CREB can sensitize prostate cancer cells to radiation and inhibit FIR-induced NED.

- We have also demonstrated that targeting PRMT5 not only increases radiation-induced cell death during the first two weeks, but also inhibits radiation-induced NED during the second two weeks. These results together suggest that targeting PRMT5 may be developed as a novel approach for prostate cancer radiosensitization.

- We have also demonstrated that PRMT5 may methylate CREB and facilitate IR-induced CREB activation during FIR-induced NED.

- We have demonstrated that PRMT5 is an epigenetic activator of AR transcription. Given that ADT is used as a radiation sensitization approach for prostate cancer treatment, this finding further support our hypothesis that targeting PRMT5 is an effective radiation sensitization approach.

- We have also found that PRMT5 may act as a master epigenetic regulation of radiation-induced DSBs repair via regulation of target gene expression involved in both HR and NHEJ.

- We have shown that PRMT5 expression can be regulated at the transcriptional level by NF-YA in prostate cancer cells.

- We have also shown that PRMT5 expression is subjected to post-translational regulation and that the E3 ligase CHIP is responsible for regulation of PRMT5 expression in prostate cancer cells.

5. Conclusion

Under the support of this prostate cancer Idea Development Award, we have made the following important discoveries. **First**, using CREB targeting as a model system, we have demonstrated that FIR-induced NED constitutes two distinct phases: acquisition of radiation resistance during the first two weeks and NED during the second two weeks. Further, we have demonstrated that targeting either phase can sensitize prostate cancer cells to radiation, and targeting both phases is a potent radiation sensitization approach. **Second**, we have identified PRMT5 as a critical upstream regulator of FIR-induced CREB activation and NED. Similarly, targeting PRMT5 during either of the two phases can sensitize prostate cancer cells to radiation. **Third**, we have discovered that PRMT5 is a novel epigenetic activator of AR transcription in prostate cancer cells and PRMTT5 expression correlates positively with AR expression in prostate cancer tissues. **Fourth**, we have generated preliminary data showing that PRMT5 may act as a master epigenetic regulator of IR-induced DNA DSBs via regulation of target gene expression in both homologous recombination and non-homologous end joining as well as G2 arrest. **Fifth**, we have also investigated the regulation of PRMT5 expression in prostate cancer cells and discovered that PRMT5 expression is transcriptionally regulated by NF-Y and post-translationally regulated by CHIP in prostate cancer cells. Given the fact that PRMT5 regulates AR expression, DNA damage response and NED, our findings collectively suggest that targeting PRMT5 is an effective radiation sensitization approach not only for prostate cancer but also for other human cancers. We will also investigate whether targeting PRMT5 is an effective radiosensitization and chemosensitization approach for other human cancers. To further develop therapeutic agents targeting PRMT5, we have been collaborating Dr. Chenglong Li at University of Florida to develop a novel type of PRMT5 inhibitor to inhibit the binding of PRMT5 to both substrates and cofactors. This novel inhibitor BLL3.3 has been proven to effectively inhibit AR expression.

Under the support of this award along with another 2011 Idea Development Award, we have published 5 articles, and presented our work in 11 conferences and at other institutions. We have also generated preliminary data that enable us to successfully acquire a new Idea Development Award from PCRP (2015) and a RO1 from NIH/NCI (2017-2022). In addition, 32 students have conducted research in the lab through their thesis research, rotations and undergraduate research, and 3 visiting scholars and one lab technician have received training through working on the project.

6. Publications, Abstracts, and Presentations

(1) Publications

a. Zhang HT, Zhang D, Zha ZG and Hu CD. Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells. Biochim Biophys Acta, 1839:1330-1340 (2014).
b. Suarez CD, Deng X, and Hu CD Targeting CREB inhibits radiation-induced neuroendocrine differentiation and increases radiation-induced cell death in prostate cancer cells. *Am J Cancer Res*, 4:850-861 (2014).
c. Hu CD, Choo R, and Huang J. Neuroendocrine differentiation in prostate cancer: a mechanism of radioresistance and treatment failure. Front Oncol, 5:90. Doi: 10.3389/fonc.2015.00090 (2015).
d. Zhang, H., Zeng, L., Tao, A.W., Zha, Z., and Hu, C.D*. The E3 ubiquitin ligase CHIP mediates ubiquitination and protesomal degradation of PRMT5. *Biochem Biophys Acta*, 1863:336-346 (2016).
e. Deng, X., Shao, G., Zhang, H.T., Li, C., Zhang, D., Cheng, L., Elzey, B.D., Pili, R., Ratliff, T.L., Huang, J., Hu, C.D. Protein arginine methyltransferase 5 functions as an epigenetic activator of the androgen receptor to promote prostate cancer cell growth. *Oncogene*, 36:1223-1231 (2017).

(2) Presentations

a. Neuroendocrine differentiation: An emerging mechanism of radiation resistance in prostate cancer
   Place: University of Colorado Cancer Center
   Date: September 6th, 2017
b. Neuroendocrine differentiation: An emerging mechanism of therapeutic resistance in prostate cancer
   Place: Department of Pathology, Northwestern University
   Date: May 15, 2017
c. Protein arginine methyltransferase 5 (PRMT5) is a novel epigenetic regulator of androgen receptor in prostate cancer
   Place: 2016 AUA meeting
   Date: May 10, 2016
d. Advances in prostate cancer diagnosis and treatment- A comparative analysis between China and America
   Place: Tongling First People's Hospital
   Date: January 5th, 2015
e. Mechanisms and targeting of therapy-resistant prostate caner
   Place: Purdue-IU Cancer Retreat
   Date: May 1st, 2015
f. Targeting protein arginine methyltransferase 5 as a novel approach to radiosensitize prostate cancer cells
   Place: Fort Lauderdale. 61st Radiation Research Society Annual meeting
   Date: September 19-22, 2015
g. Targeting PRMT5 for prostate cancer radiosensitization
   Place: Jinan University the first Affiliated Hospital

Time: December 29th, 2014

h. Mechanisms and targeting of radiotherapy-induced neuroendocrine differentiation for prostate cancer treatment
   Place: Mayo Clinic, Department of Radiation Oncology
   Date: May 18, 2014
i. Targeting PRMT5 for prostate cancer ratiosensitization
   Place: Jinan University College of Medicine
   Date: December 29, 2014
j. Targeting neuroendocrine differentiation as a novel radiosensitization approach for prostate cancer treatment
   Place: UCLA, Department of Pathology
   Date: February 27, 2014
k. Development of radiosentizers: An urgent need for prostate cancer radiotherapy
   Place: Cancer Hospital, Hefei Institutes of Physical Science Chinese Academy of Sciences
   Date: October 9th. 2013

7. Inventions, Patents and Licenses

None

8. Reportable Outcomes

a. All five published work listed above in Publications is available and have been deposited to NIH PubMed central.
b. The newly developed PRMT5 inhibitor BLL3.3. by the co-investigator Dr. Chenglong Li has been preclinically evaluated in prostate cancer cells and normal cells. These data have been included in the recently published Oncogene paper. This will advance further development and preclinical evaluation of PRMT5 inhibitors

9. Other Achievements

a. Establishment of doxycycline-inducible stable cell lines to knockdown PRMT5, Sp1, NFY-A (LNCaP-shPRMT5, DU-145-shPRMT5, C4-2-shPRMT5, RWPE1-shPRMT5, LNCaP-shSp1, LNCaP-shNFY-A). These research materials will be available for scientific community.
b. Establishment of radiation-resistant sublines after 40- and 70 Gy of FIR using LNCaP and DU-145 cells as reported here. The work reporting these development will be submitted for publication and will be made available for scientific community.
c. Construction of plasmids: There are many plasmids constructed throughout this work and all plasmids that have been published in the 4 research articles are available for scientific community, and some plasmids have already been distributed to several labs (e.g., PRMT5 luciferase reporter gene constructs from *BBA*, 2014, and PRMT5 knockdown plasmids).
d. Training of 4 graduate students awarded degrees: Chris Suarez (Ph.D. awarded in Dec 2012, currently Field Scientist at Corning), Chi-chao Hsu (Ph.D. awarded in Dec 2012, currently postdoc at University of Texas M.D. Anderson Cancer Center), Gyeon Oh (M.S. awarded in May 2015, currently Ph.D. at University of Kentucky), Sarah Kelsey (M.S. awarded in Aug 2016, currently employed by a Clinical Diagnostic company in Cincinnati).

e. Training of 2 current graduate students in the lab: Two graduate students Jake Owens (4rd year) and Elena Beketova (3rd year) have been partially working on the project.
f. Training of a visiting graduate student: Huantin Zhang, a visiting graduate student from Jinan University, worked on the transcriptional regulation of PRMT5, has published his work in BBA (2014, 2016). He was awarded Ph.D. in July 2015, and currently is working as a postdoc at Jinan University.
g. Training of 2 visiting scholars: Yihang Wu, a visiting professor from Jiliang University, China, was studying in the lab and participating in the project. He received training in molecular biology and returned to his home institution on August 17, 2015. Genbao Shao, a visiting associate professor, received training in molecular biology and prostate cancer research from Feb 2015 to Jan 2016. He is also the second author of the Oncogene paper.
h. Training of 17 rotation graduate students (4-8 weeks per rotation): Sarah Kelsey (employed by a Clinical Diagnostic Lab in Cincinnati), Lama Abdullah Alabdi (graduate student at Purdue), Jake Owens (graduate student in the lab), Mitul Patel (graduate student at Purdue), Julio Grimmn De Guibert (graduate student at Purdue), Elena Beketova (graduate student in the lab), Rui Gan (graduate student at Purdue), Aindrilla Saha (graduate student at Purdue), Maurina Aranda (graduate student at Purdue), Ziyun Ding (graduate student at Purdue), Rmah Ali (graduate student at Purdue), Yi Yang (graduate student at Purdue), Hao Chen (PULSe graduate student), Hanrui Wu (PULSe graduate student), Bao Cai (MCMP graduate student), Shiqi Tang (MCMP graduate student), and Sijie Wang (MCMP graduate student). Three of them joined the lab for MS and Ph.D. study.
i. Training of 9 undergraduate students and pharmacy students for undergraduate research (at least one semester): Athena He (Aug 2015-July 2016, prepharmacy student at Purdue), Jialu Deng (Summer 2014, Pharm.D. awarded in May 2015, employed in CVS in California), Limin Zhang (Summer 2014, Pharm.D awarded in May 2015, currently employed in Walmart, West Lafayette, IN.), George Crabtree (Aug 2014-Dec 2015, currently pharmacy student at Purdue), Yadi Xu (Spring 2013, BS awarded in May 2014, currently employed as research assistant at NIH), Myra Fu (Spring 2015, currently pharmacy student at Purdue), Athena He (Fall 2015-Spring 2016), Jonathan Malola (Fall 2016-present)
j. Funding received based on the work supported by this award:
(1): DoD PCRP Idea Development Award (PC150697): Co-targeting of androgen synthesis and androgen receptor expression as a novel treatment for castration resistant prostate cancer
(2) NIH/NCI RO1: Role and targeting of PRMT5 in prostate cancer (2017-2022)

10. References

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2015 RRS Abstract

Targeting protein arginine methyltransferase 5 as a novel approach to radiosensitize prostate cancer cells

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Radiotherapy is a standard of care for prostate cancer patients, particularly those with high-risk diseases. Despite that the majority of patients can be cured by radiotherapy, recurrence unfortunately occurs in some patients, ranging from 10% of patients with low-risk diseases up to 50% of patients with high-risk diseases. The factors that contribute to these treatment failures can be broadly classified into intrinsic radioresistance in a subset of tumor cells prior to the treatment and acquired radioresistance during the course of treatment. Recently, we have demonstrated that ionizing radiation (IR) can induce neuroendocrine differentiation (NED) in prostate cancer cells, a process by which prostate cancer cells transdifferentiate into neuroendocrine-like (NE-like cells). NE-like cells lack the expression of androgen receptor and are apoptosis resistant, and the NED process is reversible. Accumulating evidence suggests that NED is associated with disease progression and poor prognosis. Molecular analysis has revealed that the transcription factor cAMP response element binding (CREB) protein mediates IR-induced NED, and that targeting CREB can sensitize prostate cancer cells to radiation. To identify upstream regulators of CREB, we have performed mass spectrometry to identify interacting proteins of CREB in LNCaP cells. Protein arginine methyltransferase 5 (PRMT5), an epigenetic enzyme involved in regulation of many cellular processes including DNA repair, was identified as one of the CREB interacting proteins. Interestingly, IR increases the expression of PRMT5 and IR-induced PRMT5 expression is required for the repair of radiation-induced DNA damages. Further, PRMT5 interacts with CREB in the cytoplasm and mediates IR-induced CREB activation and NED. Importantly, knockdown of PRMT5 sensitizes prostate cancer cells to radiation in clonogenic assays. These findings suggest that targeting PRMT5 is an effective approach to sensitize prostate cancer cells to radiation.

2016 AUA Abstract (16-6245)

Protein arginine methyltransferase 5 is a novel epigenetic regulator of androgen receptor in prostate cancer

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Introduction and Objective: Protein arginine methyltransferase 5 (PRMT5) is an emerging epigenetic enzyme that regulates many cellular processes by epigenetic control of target gene expression. Several reports showed that PRMT5 is overexpressed in several human cancers and that its overexpression appears to promote cancer cell growth. The objective of this study is to determine the role of PRMT5 in regulation of prostate cancer cell proliferation in vitro and tumor growth in mice.

Methods: Immunohistochemistry was used to determine the expression level of PRMT5 in prostate cancer tissues. Short hairpin RNA (shRNA) was used to knock down PRMT5. Western blotting and real-time PCR were used to determine the expression level of PRMT5 in prostate cancer cells at the protein and mRNA levels. To determine how PRMT5 regulates AR expression, luciferase reporter gene, chromatin immunoprecipitation (ChIP), coimmunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC) assays were used.

Results: To determine whether PRMT5 regulates prostate cancer cell proliferation, we knocked down PRMT5 by shRNA in multiple prostate cancer cell lines, and observed that PRMT5 regulates prostate cancer cell proliferation in an AR-dependent manner in both hormone naïve and castration-resistant prostate cancer cells. Molecular analyses revealed that PRMT5 interacts with the transcription factor SP1 on the AR promoter and epigenetically regulates AR transcription. Significantly, PRMT5 is overexpressed in prostate cancer tissues when compared with benign prostatic hypertrophy, and its expression positively correlates with AR expression in these tissues. Remarkably, knockdown of PRMT5 in LNCaP prostate cancer cells completely suppresses xenograft tumor growth in mice.

Conclusion: We have demonstrated that PRMT5 is a novel epigenetic regulator of AR in prostate cancer cells. Given that AR is the driving force of prostate cancer development and progression, our results suggest that targeting PRMT5 may be explored as a novel therapeutic approach via inhibition or elimination of AR expression.

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Original Article

Targeting CREB inhibits radiation-induced neuroendocrine differentiation and increases radiation-induced cell death in prostate cancer cells

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Abstract: Neuroendocrine differentiation (NED) is a process by which prostate cancer cells transdifferentiate into neuroendocrine-like (NE-like) cancer cells. Accumulated evidence suggests that NED is associated with disease progression and therapy resistance in prostate cancer patients. We previously reported that by mimicking a clinical radiotherapy protocol, fractionated ionizing radiation (FIR) induces NED in prostate cancer cells. Interestingly, FIR-induced NED constitutes two distinct phases: a radioresistance phase in which a fraction of cells selectively survive during the first two week irradiation, and a neuroendocrine differentiation phase in which surviving cells differentiate into NE-like cancer cells during the second two week irradiation. We have also observed increased activation of the transcription factor cAMP response element binding (CREB) protein during the course of FIR-induced NED. To determine whether targeting NED can be explored as a radiosensitization approach, we employed two CREB targeting strategies, CREB knockdown and overexpression of ACREB, a dominant-negative mutant of CREB, to target both phases. Our results showed that ACREB expression increased FIR-induced cell death and sensitized prostate cancer cells to radiation. Consistent with this, knockdown of CREB also inhibited FIR-induced NED and sensitized prostate cancer cells to radiation. Molecular analysis suggests that CREB targeting primarily increases radiation-induced premitotic apoptosis. Taken together, our results suggest that targeting NED could be developed as a radiosensitization approach for prostate cancer radiotherapy.

Keywords: Prostate cancer, radiosensitization, neuroendocrine differentiation, NED, CREB

Introduction

Prostate cancer is the second-leading cause of cancer death in American men [1]. Approximately 15-20% of prostate cancer patients were diagnosed with high-risk cancer that is either clinical stage T3, a Gleason score of 8-10 or prostate specific antigen > 20 ng/ml [2]. Radiotherapy (RT) plus androgen deprivation therapy (ADT) is the standard treatment for these patients [2-4]. However, 30-60% of patients with high-risk cancer still experience biochemical recurrence within 5 years [5-7]. Thus, high-risk prostate cancer represents a therapeutic challenge for prostate cancer management.

Neuroendocrine differentiation (NED) in prostate cancer is a process by which prostate cancer cells transdifferentiate into neuroendocrine (NE)-like prostate cancer cells [8]. NE cells are one type of prostatic epithelial cells that constitutes less than 1% of total epithelial cells. However, increased numbers of NE-like prostate cancer cells have been observed in prostate cancer patients [9-11]. Accumulated evidence suggests that NED is associated with disease progression, androgen-independent growth and poor prognosis in prostate cancer patients [8, 12-14], and NED can be induced by a number of stimuli including ADT [15-17] and chemotherapy [18]. We previously reported that fractionated ionizing radiation (FIR) induces NED in LNCaP prostate cancer cells [19] and this finding has recently been extended to DU-145 and PC-3 cells and in LNCaP xenograft tumors [20]. Importantly, FIR-induced NED is a reversible process and isolated radiation-resistant sublines are cross-resistant to radiation, androgen depletion and docetaxel treatments

[19]. Given that our recent pilot clinical study has shown 4 out of 9 patients may undergo NED [20], it is very likely that RT-induced NED may contribute to radioresistance and tumor recurrence in prostate cancer patients.

The mechanisms underlying NED remain to be defined [8]. It appears that distinct mechanisms are involved in NED induced by different stimuli [8, 14, 21]. We found that FIR-induced NED correlates with increased phosphorylation of cAMP response element binding (CREB) protein at Ser133 [19] , an activating phosphorylation by many protein kinases [22]. CREB, a member of the ATF-1/CREM/CREB basic region leucine zipper transcription factor family, functions as a homodimer or heterodimer with other ATF-1/CREM/CREB family members to regulate transcription of target genes responsible for a wide range of cellular processes [23]. Studies have established a role for CREB in several human cancers [24-26]. In prostate cancer, increased expression of RGS17 enhances CREB phosphorylation to maintain tumor cell proliferation [27]. CREB activation has also been linked to aberrant expression of vascular endothelial growth factor (VEGF) and the resulting predisposition to bone metastasis [28]. In the present study, we employed a dominant negative CREB and CREB knockdown approaches to inhibiting CREB activity, and demonstrated that targeting FIR-induced NED is an effective approach to sensitizing prostate cancer cells to radiation.

Materials and methods

Establishment of stable cell lines for fractionated FIR treatment

Prostate cancer cell lines were maintained and treated with FIR (2 Gy/day, 5 days/week) as previously reported [19, 20]. The tetracycline/doxycycline inducible pcDNA4-TO system (Invitrogen) was used to establish stable cell lines (LNCaP-HA-ACREB#1-4) to express ACREB [19]. The tetracycline/doxycycline inducible lentiviral system to express short hairpin RNA (shRNA) or scrambled control (SC) was utilized to knock down CREB with pLK0.1-Tet-On (Addgene plasmid 21915). The oligonucleotides were selected using validated sequences from Sigma Aldrich and named using the last three digits corresponding to the Sigma TRCN sequence number (TRCN0000007308, TRCN0000226467, TRCN0000226468, TRCN0000226469). Lentiviral packaging using pLK0.1-CREB shRNA or pLVX-ACREB (Clontech) in HEK293T cells and establishment of prostate cancer stable cell lines expressing ACREB or CREB shRNAs were performed as reported previously [29].

MTT assay

LNCaP-HA-ACREB#1 cells were seeded in triplicate in 48-well plates at a density of 2×10^4 cells/ml. Tetracycline (5 μg/ml) was added to induce expression of HA-ACREB for 24 hours before subjecting to FIR. Medium was changed after 3 days and tetracycline was replenished. After achieving the desired dose of FIR, medium was removed from wells and 70 μl of MTT reagent was added. Cells were incubated at 37°C, 5% CO_2 for 4 hours followed by addition of 130 μl of DMSO. Plates were shaken, incubated for an additional 10 min at 37°C, 5% CO_2 and read on Biotek Synergy 4 plate reader at 570 and 700 nm. Results were from three independent experiments, and two-way ANOVA analysis was performed to determine the statistical significance.

Cell cycle analysis via flow cytometry

LNCaP-HA-ACREB#1 cells were treated with tetracycline (5 μg/ml) to induce expression of HA-ACREB for 24 hours, followed by FIR. Medium with fresh tetracycline was changed every 3 days. Cells were harvested, fixed in 70% ethanol and temporarily stored at 4°C, and then resuspended in 500 μl freshly prepared propidium iodide (PI) working solution prior to flow cytometry analysis. Data was collected on Beckman Coulter FC 500 flow cytometer and analysis was completed using FlowJo software (Treestar, Inc., Ashland, OR). Three independent experiments were performed and two-way ANOVA analysis was performed to determine the statistical significance.

Immunoblotting of γH2AX, PARP cleavage, and LC-3 cleavage

LNCaP-HA-ACREB#1 cells were treated with doxycycline or water for 48 hours, and then subjected to FIR (2 Gy/day). Irradiated cells including floating cells were harvested 24 hours after the last IR treatment and total lysate was prepared for immunoblotting analysis using antibodies against γH2AX (Cell Signaling Technology, #9718), cleaved poly ADP ribose polymerase (PARP) (BD Pharmingen, #556494),

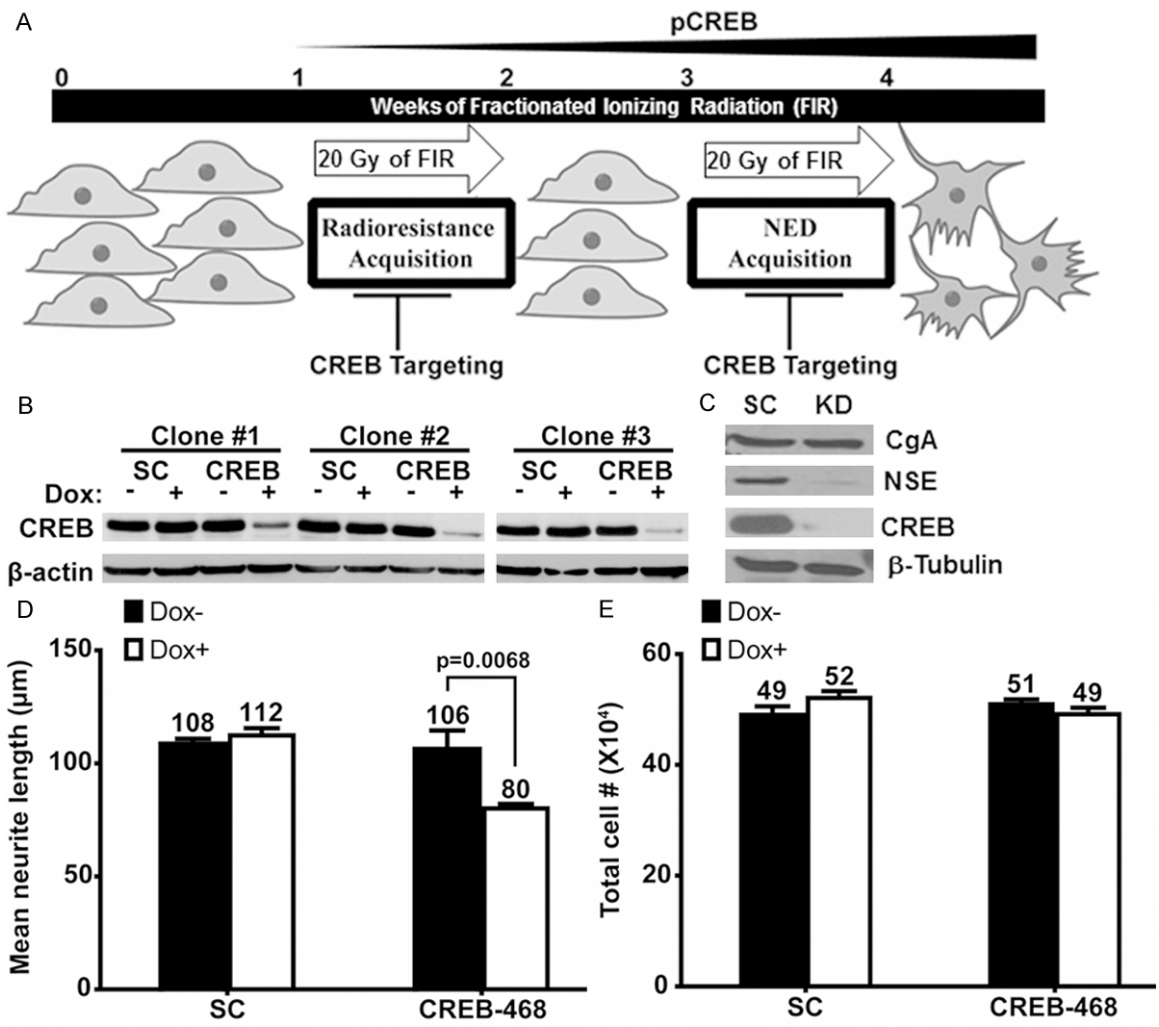

Figure 1. CREB knockdown inhibits IR-induced neuroendocrine differentiation. A: Shown is the model system depicting FIR-induced NED. FIR-induced NED constitutes two phases: radioresistance during the first two weeks and NED acquisition during the second two weeks. Increased phosphorylated CREB (pCREB) was observed during the course of FIR-induced NED. B: Three independently transduced LNCaP stable cell lines show efficient knockdown using the CREB-468 shRNA (CREB) plasmid when compared with the scrambled control (SC). C: Effect of CREB knockdown on the expression of CgA and NSE after 40 Gy of FIR. D: The established three stable cell lines expressing CREB shRNA (CREB-468) or SC were subjected to FIR (2 Gy/day, 5 days/week) for a total dose of 40 Gy. At the end of treatment, images were captured and neurite extension was quantified. Results presented are mean of the three independent experiments. Error bars represent standard deviation. E: Similar experiments were conducted as described in D and the total number of surviving cells were counted after trypsinization.

and microtubule-associated protein 1A/1B-light chain 3 (LC-3) (Novus Biologicals, NB100-2220) to determine the underlying mechanisms of cell death. To determine whether pre-mitotic or post-mitotic cell death occurred in ACREB expressing cells, cells were induced to express ACREB for 48 hours and then subjected to a single dose of 2 Gy ionizing radiation (IR). The total cell lysate was prepared 4 hours after the irradiation for immunoblotting analysis of PARP cleavage. For preparation of total cell lysate at 24 hours after the irradiation, floating cells were removed by changing the medium at 12 hours, and the total cell lysate was prepared for PARP cleavage analysis at 24 hours after the irradiation treatment.

Immunofluorescence analysis of activated caspse-3

To quantify the number of cells with activated caspase-3, cells were first induced to express HA-ACREB with or without doxycycline, and then subjected to 2 Gy of IR or without IR treatment, followed by fixation and staining with

anti-cleaved caspase-3 antibody (Cell Signaling Technology, #9664) and a secondary Texas Red-conjugated anti-rabbit antibody and 4', 6-diamidino-2-phenylindole (DAPI). The percentage of activated caspase-3 positive cells was calculated by dividing the number of cells stained red by the total number of cells counted (DAPI positive). For each experiment, at least 120 cells were counted, and three independent experiments were conducted. Results were analyzed using student's *t*-test.

Clonogenic assays

LNCaP-HA-ACREB#1 or LNCaP-CREB shRNA#-468 cells or the control cell lines were first induced with or without doxycycline (1 μg/ml) for 48 hours (for ACREB) or 72 hours (for shRNAs), and then subjected to a single exposure of different doses of IR. Irradiated cells were trypsinized immediately and various numbers of cells were seeded in 6-well plates and cultured in complete medium with or without doxycycline for two weeks. At the end of experiments, the number of colonies was counted and surviving fractions were calculated as described [30]. Student's *t* test was used to determine the statistical significance.

Quantification of neurite extension and immunoblotting analysis of chromogranin A and neuron specific enolase

LNCaP-HA-ACREB stable cell lines were subjected to 40 Gy of FIR, and images were captured using a Nikon TE-2000 inverted epifluorescence microscope with CoolSnap CCD camera. Image processing and analysis was completed using ImageJ software modified by the McMaster Biophotonics Facility in Ontario, Canada (revision 1.44k). Neurite extension was quantified using the ImageJ plugin NeuronJ from Erik Meijering [31]. Quantification was performed using 10 image fields per condition. Results presented were from three independent experiments and two-tailed *t*-test was used to determine the statistical significance. The expression of chromogranin A (CgA) and neuron specific enolase (NSE) was similarly examined as reported previously [19].

Results

CREB knockdown inhibits FIR-induced neurite extension and NSE expression

To dissect the role of CREB in FIR-induced NED in prostate cancer cells (**Figure 1A**), we employed a lentivirus-based tetracycline-inducible knockdown system to generate four LNCaP cell lines containing stably integrated CREB shRNA expression plasmid. Screening of these four cell lines showed variable knockdown efficiency with CREB #468 achieving approximately 85% knockdown efficiency (data not shown). We then used CREB #468 to conduct three independent transductions to generate stable LNCaP cell lines that had comparable knockdown efficiency (**Figure 1B**). To determine the effect of CREB knockdown on FIR-induced NED, we performed 40 Gy of FIR and measured the expression of CgA and NSE. While we observed a dramatic inhibition of NSE expression when compared with SC, the expression level of CgA was not altered by CREB knockdown (**Figure 1C**). To quantify the effect of CREB knockdown on neurite extension and cell viability, we used the established three independent sublines to perform 40 Gy of FIR. Like the expression of a non-phosphorylatable CREB (S133A) [19], we observed that CREB knockdown significantly decreased neurite extension (**Figure 1D**). However, CREB knockdown failed to increase FIR-induced cell death (**Figure 1E**). The inability of CREB knockdown to increase FIR-induced cell death is not due to the selection of established stable clones as transient expression of CREB shRNAs also failed to increase FIR-induced cell death after 10 Gy of FIR (unpublished observation) and another CREB knockdown construct targeting a different region of the CREB coding sequence yielded similar results (data not shown).

Expression of a dominant negative CREB increases FIR-induced cell death

Our observation that CREB knockdown did not increase FIR-induced cell death is surprising, given that CREB phosphorylation was induced even after 10 Gy of FIR [19]. Because there are at least 3 members in the CREB/CREM/ATF-1 family that can form dimers with CREB to regulate target gene transcription [22], we reasoned that these family members might compensate for the reduction of CREB to regulate expression of target genes essential for cell survival. Alternatively, the residual amount of CREB might be sufficient to regulate expression of these target genes. To circumvent this potential problem, we used ACREB, a dominant negative CREB, in which the leucine zipper region of CREB is used and the basic region is replaced with acidic amino acid residues [32], to evalu-

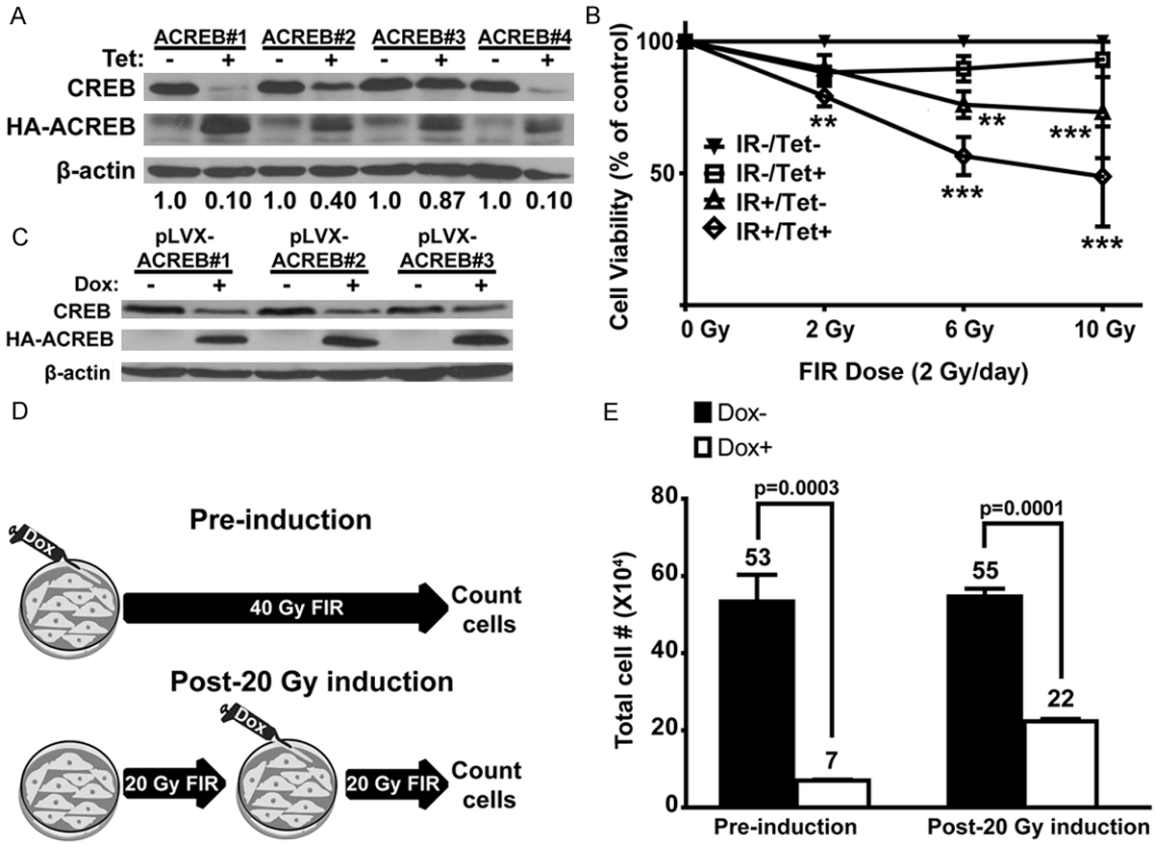

Figure 2. Expression of a dominant-negative CREB increases radiation-induced cell death. (A) Establishment of 4 independently isolated stable and tetracycline-inducible LNCaP clones expressing HA-ACREB using the pcDNA4TO expression system (Invitrogen). Induction of HA-ACREB inhibited auto-regulation of CREB. The numbers indicate relative level of tetracycline-induced (Tet+) CREB expression when compared with non-induced (Tet-). (B) The stable cell line ACREB#1 in A was subjected to the indicated doses of FIR and cell viability was analyzed using the MTT assay. $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Establishment of 3 stable and doxycycline-inducible cell lines expressing HA-ACREB by 3 independent lentiviral transductions using the pLVX expression system (Clontech). All three cell lines exhibit comparable induction of HA-ACREB and down-regulation of CREB by doxycycline (Dox+). (D) Shown are two experimental designs to determine the effect of HA-ACREB expression on cell survival shown in (E). HA-ACREB was induced by Dox during the entire 40 Gy of FIR (Pre-induction) or during NED acquisition phase only (Post-20 Gy induction). (E) Quantified total number of viable cells after 40 Gy of FIR.

ate the role of CREB in FIR-induced NED. Because ACREB retains the ability to dimerize with endogenous CREB and other CREB dimerization partners but cannot bind DNA, overexpressed ACREB can efficiently inhibit transcription of CREB target genes [32, 33]. For this purpose, we established stable, tetracycline inducible, LNCaP cell lines to express ACREB as a hemagglutinin (HA) fusion protein. Four individual clones were isolated, and these clones exhibited variable expression of HA-ACREB. Because CREB can autoregulate its own transcription [34], these clones also demonstrated unique effects on CREB expression (**Figure 2A**). Notably, induction of ACREB in clones #1 and #4 reduced CREB by 90%. Consistent with the expression level of ACREB and the down-regulation of CREB in these clones, induction of ACREB in clone #1 increased FIR-induced cell killing after 10 Gy of FIR (**Figure 2B**) whereas induction of ACREB in clones #2 and #3 had little effect on FIR-induced cell killing (unpublished observation). These results not only demonstrate that ACREB is a potent inhibitor of CREB activity but also suggest that CREB plays a role in conferring radioresistance even during the first week of irradiation.

Long-term expression of ACREB dramatically increases FIR-induced cell killing

To determine the effect of long-term expression of ACREB on FIR-induced cell death, we performed long-term FIR treatment. While attempt-

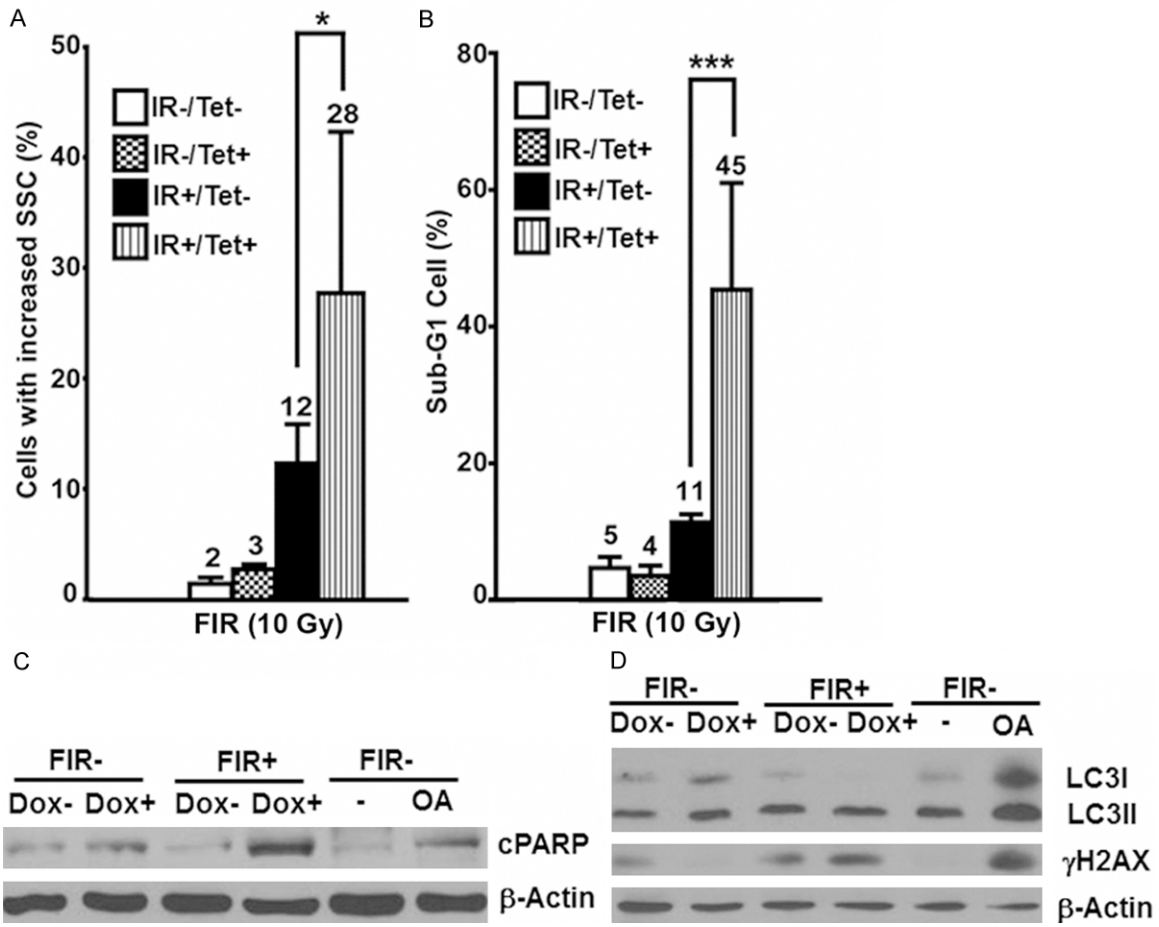

Figure 3. ACREB expression increases IR-induced apoptosis in LNCaP cells. A: LNCaP-HA-ACREB#1 cells were subjected to 10 Gy of FIR (IR+) or without IR treatment (IR-) in the absence (Tet-) or presence (Tet+) of tetracycline. Cell granularity was analyzed by flow cytometry. B: Similar experiments in A were conducted and the number of sub-G1 cells was analyzed by flow cytometry. C and D: LNCaP-HA-ACREB#1 cells were subjected to 10 Gy of FIR (FIR+) or without FIR (FIR-) in the absence (Dox–) or presence of doxycycline (Dox+), and cell lysate was prepared 24 hours after the last irradiation for immunoblotting analysis of cleaved PARP (cPARP), LC3I and LC3II, and γH2AX. As a positive control, cells were treated with 50 nM of okadaic acid (OA) or DMSO (-) for 24 hours. $^{*}P < 0.05$; $^{***}P < 0.001$.

ing these experiments, using clones derived from the Invitrogen pcDNA6/TR/pcDNA4/TO expression system, there was excessive cell death under both induced and non-induced conditions, which is likely due to the effect of radiation-induced damage to the DNA encoding the tetracycline-resistance operon [35]. To overcome this problem, we utilized the Clontech pLVX-Tet-On lentiviral expression system that does not rely on the dissociation of the Tet repressor protein from the tetracycline-resistance operon [36]. Stable clones were prepared using three independent transductions and induction of ACREB sufficiently down-regulated the expression of CREB in each cell line (**Figure 2C**). To separate the role of CREB in both phases, we specifically induced ACREB expression during the NED phase only (weeks 3 and 4, post-20 Gy induction) and during the entire 4 weeks (pre-induction) to assess the impact of ACREB expression on the total number of viable cells at the end of 40 Gy FIR (**Figure 2D**). Induction of ACREB during the entire FIR treatment period resulted in a 7.6-fold reduction in cell number, and induction of ACREB during the NED phase also resulted in a 2.5-fold reduction (**Figure 2E**). These results suggest that CREB plays a critical role in the acquisition of radioresistance and the acquisition of NED during the process of FIR-induced NED.

ACREB expression increases radiation-induced apoptosis

The transcriptional activity of CREB is required for regulation of many cellular processes includ-

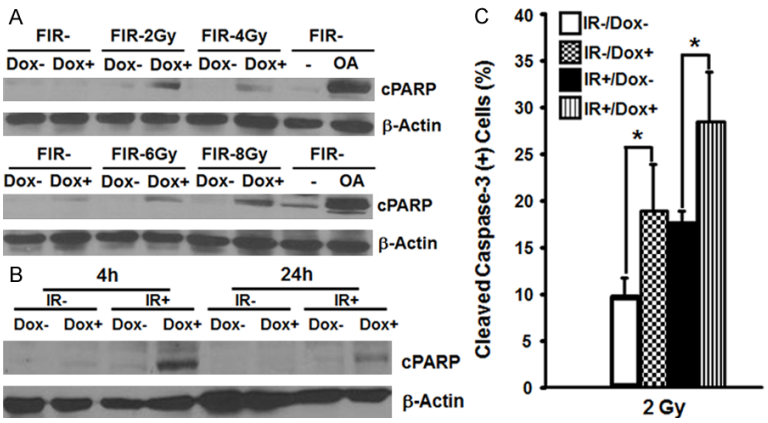

Figure 4. ACREB expression induces pre-mitotic and post-mitotic apoptosis. A: LNCaP-HA-ACREB#1 cells were induced by doxycycline (Dox+) to express ACREB for 48 hours or without induction (Dox-), and then subjected to FIR for the indicated doses. Cell lysate was prepared 24 hours after the last irradiation treatment and cleaved PARP (cPARP) was analyzed by immunoblotting. As a positive control, cells were treated with 50 nM of okadaic acid (OA) or DMSO (-) for 24 hours. B: LNCaP-HA-ACREB#1 cells were induced to express ACREB by doxycycline (Dox+) for 48 hours or without induction (Dox-), followed by a single exposure to 2 Gy ionizing radiation (IR+) or without irradiation (IR-). Cell lysate was prepared 4 and 24 hours after the irradiation for immunoblotting analysis of cPARP. C: LNCaP-HA-ACREB#1 cells were similarly treated in B, and caspase-3 activation was assayed by immunostaining of cleaved caspase-3 at 4 hours after the irradiation.

ing cell cycle, apoptosis, cell proliferation and differentiation [23]. To uncover the molecular mechanism by which ACREB expression increases IR-induced cell death, we examined the effect of ACREB expression on cell cycle, apoptosis, autophagy and DNA damage. Flow cytometry analysis revealed that FIR treatment in ACREB expressing cells exhibited increased granularity after 10 Gy of FIR. This granular population of cells increased by 2.3 fold when compared with FIR treated LNCaP not expressing ACREB (**Figure 3A**). Flow cytometry analysis using PI showed a 4-fold increase in the sub-G1 population in ACREB expressing cells treated with 10 Gy of FIR (**Figure 3B**). No significant difference in other phases of cell cycle was observed (data not shown). These results suggest that ACREB expression increases FIR-induced cell death. Because increased granularity can be associated with events such as autophagy [37] and apoptosis [38], we examined their involvement in ACREB-induced radiosensitivity. We harvested all floating and adherent cells after 10 Gy of FIR to measure PARP cleavage, and confirmed that ACREB expression indeed increased PARP cleavage (**Figure 3C**). We also performed immunoblotting analysis of LC3. Conversion of the cytosolic LC3I into autophagosome-associated LC3II allows assessment of autophagy via immunoblotting. However, ACREB induction did not increase the amount of FIR-induced LC3II nor the ratio of LC3II/LC3I (**Figure 3D**). However, ACREB expression slightly increased FIR-induced γH2AX level (**Figure 3D**).

Since we observed increased cell death with increased doses of FIR, we next determined whether this correlates with the extent of apoptosis by measuring PARP cleavage after various doses of FIR. Although ACREB expression increased the amount of cleaved PARP in all doses, there was no significant increase in cleaved PARP in higher doses (**Figure 4A**). Because we prepared total cell lysate for immunoblotting analysis of PARP cleavage 24 hours after the last irradiation of the indicated doses, these results suggest that apoptosis likely occurs within 24 hours.

Radiation-induced cell death can occur as pre-mitotic and post-mitotic cell death [39]. The former usually occurs within 4-5 hours whereas the latter occurs after 24 hours. To know whether ACREB expression increases radiation-induced pre-mitotic cell death, we performed a single dose IR and harvested cells at 4 and 24 hours to examine the level of PARP cleavage. We observed increased cell death at 4 hours after 2 Gy of irradiation, and some cells showed membrane blebbing, a typical feature of apoptotic cells (unpublished observations). Consistent with this, increased PARP cleavage in irradiated ACREB-expressing cells was observed (**Figure 4B**). However, we observed less cell death and PARP cleavage at 24 hours (**Figure 4B**). No increase in cell death or PARP cleavage was observed after 48 hours. These results suggest that ACREB induction may primarily induce pre-mitotic cell death. Because radiation-induced pre-mitotic cell death usually results from activation of pre-existing apoptotic machinery [39], we next examined the activation of caspase-3 by immunostaining, and observed that ACREB induction by itself appeared to slightly activate caspse-3. However, ACREB

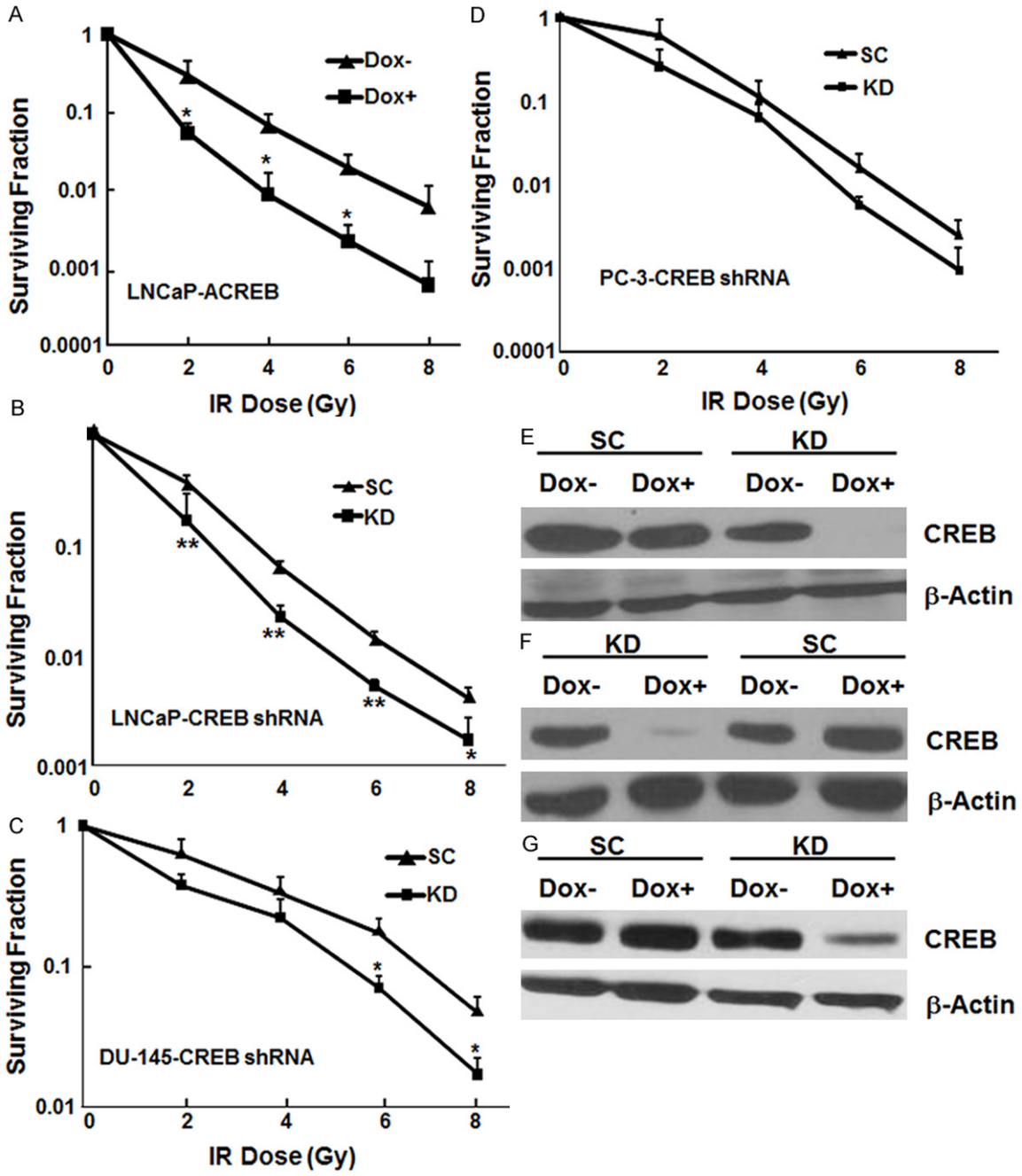

Figure 5. CREB targeting sensitizes prostate cancer cells to radiation. Indicated stable and doxycycline-inducible prostate cancer cell lines expressing HA-ACREB or CREB shRNA#468 (KD) or scrambled control (SC) were induced to express HA-ACREB for 48 hours or CREB shRNA#468 for 72 hours and then subjected to a single exposure of the indicated dose of IR, followed by seeding of various numbers of cells in 6-well plates for colony formation. Colony formation was counted 2 weeks later and survival fraction was calculated. Shown are the means from three independent experiments. $^{*}P$ <0.05; $^{**}P$ <0.01.

expression dramatically increased IR-induced caspase-3 activation (**Figure 4C**). These results collectively suggest that ACREB expression primarily increases radiation-induced pre-mitotic apoptosis via activation of caspase-3.

CREB targeting sensitizes prostate cancer cells to radiation

Our above results strongly suggest that targeting CREB signaling is an effective approach to

sensitizing prostate cancer cells to radiation. To further determine this, we used the ACREB stable cell lines to perform clonogenic assays, a standard assay for determination of radiosensitivity [30]. As shown in **Figure 5A**, induction of ACREB expression significantly sensitized LNCaP cells to radiation at all doses examined. Because the clonogenic assay utilizes a single dose treatment to assess the impact of DNA damage on cell reproduction, this is different from FIR, during which damaged DNA may be repaired by compensation for the reduction of CREB. Thus, we sought to determine whether CREB knockdown can sensitize LNCaP cells to radiation. Using the same stable cell line (#468), we observed that knockdown of CREB also sensitized LNCaP cells to radiation when compared with the scrambled control (**Figure 5B**). A similar result was observed in DU-145 (**Figure 5C**). Consistent with the lack of significant CREB activation by FIR in PC-3 cells [20], knockdown of CREB did not sensitize PC-3 to radiation (**Figure 5D**). Note that CREB expression was comparably knocked down in LNCaP (**Figure 5E**), DU-145 (**Figure 5F**) and PC-3 (**Figure 5G**) stable cell lines. Taken together, our results suggest that targeting CREB can sensitize a subset of prostate cancer cells to radiation.

Discussion

Numerous studies have demonstrated that NED is associated with disease progression and poor clinical outcome in prostate cancer patients [12]. The clinical significance of NED is further supported by the fact that ADT- and chemotherapy-induced NED correlates with poor therapeutic responses and clinical outcomes [15-18, 40]. Because NE-like cells are highly resistant to apoptosis [41] and cAMP and androgen depletion-induced NED are reversible [8, 42], it has been hypothesized that therapy-induced NED allows prostate cancer cells to survive treatment and contribute to tumor recurrence [8, 13, 14]. However, it remains unclear whether targeting therapy-induced NED can be explored to sensitize prostate cancer cells to treatments such as ADT, radiotherapy or chemotherapy. Using LNCaP cells as a model, we have demonstrated that FIR-induced NED constitutes two distinct phases: selection of radioresistant cells and NED onset (**Figure 1A**). Using two CREB targeting approaches, we provide evidence in the present study that CREB is involved in both phases and targeting CREB can increase FIR-induced cell death. In particular, expression of ACREB, a potent dominant negative CREB, increased FIR-induced cell death and sensitized LNCaP cells to FIR. Consistent with FIR-induced activation of CREB in LNCaP and DU-145 cells [20], knockdown of CREB also sensitized LNCaP and DU-145 cells to radiation. Our results suggest that inhibition of RT-induced NED may be explored to sensitize prostate cancer cells to radiotherapy. Further investigation of CREB targeting strategies [24] or identification of CREB upstream regulators will likely lead to development of novel radiosensitizers.

Although CREB signaling has been explored for its role in oncogenesis [43], the impact of CREB in cancer cell signaling has recently attracted attention. CREB targeting CRE-decoy oligonucleotides induce apoptosis in ovarian cancer cells [25] and CREB is involved in prostate cancer bone metastasis through regulation of VEGF [28]. In several studies, the dominant negative ACREB has been utilized to target CREB. One such study reported the mechanism of ACREB-induced apoptosis in rat thyroid cells [44]. It was demonstrated that S phase delay led to activation of ATR and the S-phase checkpoint without altering the regulation of pro- or anti-survival genes. These findings are consistent with the role of CREB in regulating expression of several target genes involved in the cell cycle [23]. In the present study, we demonstrate that ACREB expression efficiently sensitized LNCaP cells to FIR by increasing FIR-induced apoptosis. However, we did not see any significant S phase delay in ACREB expressing cells. It is worth noting that CREB knockdown only inhibited FIR-induced neurite outgrowth and the expression of NSE without significant effect on FIR-induced CgA expression and cell death during FIR treatment. Paradoxically, CREB knockdown was sufficient to inhibit colony formation in clonogenic assays in LNCaP and DU-145 cells. Given that CREB/CREM/ATF-1 family members can form both homodimers and heterodimers and that some target genes are regulated by these dimeric complexes [22], it is likely that the loss of CREB may be functionally compensated for by other dimeric complexes during FIR [45]. This is supported by the observation that CREB knockdown did not inhibit FIR-induced CgA expression, though CREB is a transcriptional activator

of CgA [46]. Thus, it is likely that expression of some CREB target genes critical for cell survival are not affected by CREB knockdown, but are suppressed by ACREB expression during the course of FIR treatment. Alternatively, a residual amount of CREB (e.g., 10-20%) is enough to activate the expression of target genes that confer the resistance and cell survival to FIR treatment. In agreement with this, we indeed observed that induction of ACREB in clones #1 and 4, in which CREB expression was decreased by more than 90%, efficiently increased IR-induced cell death. Conversely, induction of ACREB in clones #2 and #3, in which CREB expression was only decreased by 60% and 13% respectively, was ineffective.

Radiation-induced cell death can be a result of induction of apoptosis or autophagy [47]. It has been reported that IR-induced apoptosis and autophagy can occur in prostate cancer cells such as LNCaP [37, 48]. However, induction of ACREB did not significantly increase FIR-induced autophagy. Thus, it is unlikely that CREB is involved in the regulation of FIR-induced autophagy in LNCaP cells. Instead, we observed increased PARP cleavage and caspase-3 activation as early as 4 hours after a single exposure to IR. Interestingly, this effect appears to last for at least 24 hours. However, we failed to observe any further increase in apoptosis after 48 hours. These results collectively suggest that ACREB induction primarily increases IR-induced pre-mitotic apoptosis, and to a lesser extent post-mitotic apoptosis. Future identification of CREB target genes involved in IR-induced apoptosis and FIR-induced NED will provide new insight into the role of CREB in radioresistance and FIR-induced NED.

In conclusion, we have employed two CREB targeting approaches and demonstrated that CREB is involved in both the acquisition of radioresistance and the acquisition of NED during FIR-induced NED. In particular, expression of ACREB potently increased FIR-induced apoptosis and sensitized prostate cancer cells to radiation. Our results suggest that targeting FIR-induced NED is an effective approach to sensitizing prostate cancer cells to radiation.

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Disclosure of conflict of interest

None to declare.

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Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells

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ABSTRACT

Protein arginine methyltransferase 5 (PRMT5) symmetrically methylates arginine residues of histones and non-histone protein substrates and regulates a variety of cellular processes through epigenetic control of target gene expression or post-translational modification of signaling molecules. Recent evidence suggests that PRMT5 may function as an oncogene and its overexpression contributes to the development and progression of several human cancers. However, the mechanism underlying the regulation of PRMT5 expression in cancer cells remains largely unknown. In the present study, we have mapped the proximal promoter of PRMT5 to the −240 bp region and identified nuclear transcription factor Y (NF-Y) as a critical transcription factor that binds to the two inverted CCAAT boxes and regulates PRMT5 expression in multiple cancer cell lines. Further, we present evidence that loss of PRMT5 is responsible for cell growth inhibition induced by knockdown of NF-YA, a subunit of NF-Y that forms a heterotrimeric complex with NF-YB and NF-YC for function. Significantly, we have found that activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) in LNCaP prostate cancer cells down-regulates the expression of NF-YA and PRMT5 at the transcription level in a c-Fos-dependent manner. Given that down-regulation of several PKC isozymes is implicated in the development and progression of several human cancers, our findings suggest that the PKC-c-Fos-NF-Y signaling pathway may be responsible for PRMT5 overexpression in a subset of human cancer patients.

1. Introduction

Protein arginine methyltransferase 5 (PRMT5), a type II methyltransferase that symmetrically methylates arginine residues of histones and non-histone protein substrates [1,2], regulates a variety of cellular processes by epigenetic regulation of target gene expression and by post-translational modification of critical signaling molecules [1]. Recently, several studies have shown that PRMT5 is overexpressed in human cancers such as lung cancer [3,4], ovarian cancer [5], colorectal cancer [6], breast cancer [7], melanoma [8], leukemia and lymphoma [9,10], and glioblastoma [11]. The overexpression of PRMT5 correlates with disease progression and poor prognosis. Importantly, these studies also present evidence that silencing PRMT5 expression in these cancer cells inhibits cell proliferation and/or induces apoptosis, suggesting that PRMT5 overexpression in cancer cells plays an important role in the development and progression of human cancers. However, how PRMT5 expression is transcriptionally regulated in cancer cells has not yet been investigated.

Nuclear transcription factor Y (NF-Y) is an important transcription factor that is highly conserved across the species [12–14]. NF-Y is composed of three subunits, NF-YA, NF-YB and NF-YC, and functions as a heterotrimeric complex to bind the CCAAT box in promoter regions to regulate gene transcription. CCAAT boxes are usually positioned in either orientation between −60 and −100, and are present in almost 30% of human promoters, particularly those that drive expression of oncogenes in human cancers [15–17]. In addition, NF-Y binding sites overlap with binding sites of several other transcription factors, such as SP1, E2F1, GATA, and c-Fos, to cooperatively regulate cell growth [12,15,18]. The NF-Y transcriptional activity can be modulated by increasing DNA binding to the CCAAT boxes [19,20] or by increasing expression of the NF-YA subunit [12,21–23]. However, whether the cancer signaling regulates NF-YA expression remains unknown.

Abbreviations: PRMT5, Protein arginine methyltransferase 5; NF-Y, Nuclear transcription factor Y; PKC, Protein kinase C; AP-1, Activator protein-1; PMA, Phorbol 12-myristate 13-acetate; GFX, Bisindolylmaleimide I; TCL, Total cell lysate; CCNA2, Cyclin A2; Dox, Doxycycline; SC, Scrambled control; shRNA, Short hairpin RNA; BrdU, Bromodeoxyuridine; indel, Insertion–deletion; SNPs, Single nucleotides polymorphisms; WT, Wild-type

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Protein kinase C (PKC) is a family of serine/threonine protein kinases that regulates a wide range of cellular processes [24]. PKC isozymes can be classified into three groups including calcium-dependent "classical" cPKCs (α, βI, βII and γ), calcium-independent "novel" nPKCs (δ, ε, η and θ), and calcium-independent "atypical" aPKCs (ζ and ι/λ). Classical and novel PKC isozymes, but not atypical PKC isozymes, can be activated by diacylglycerol (DAG) and phorbol 12-myristate 13-acetate (PMA). Although it is generally thought that most PKC isozymes are overexpressed in human cancers and promote cellular transformation, proliferation, and migration, the opposite effects have also been reported [24]. This is exemplified by the use of prostate cancer cells as a model system to study distinct roles of PKC isozymes in apoptosis in prostate cancer cells [25], in which treatment of LNCaP, but not DU 145 and PC-3 cells, with PMA induces apoptosis [26]. Consistent with their differential roles in cell-based studies, the expression level of several PKC isozymes in some human cancers inversely correlates with the aggressiveness of the disease [27,28]. However, the mechanism by which down-regulation of PKC isozymes regulates cancer cell growth remains unknown.

Activator protein 1 (AP-1) is a family of dimeric transcription factors which includes c-Jun and c-Fos [29]. AP-1 was discovered as a complex of c-Fos/c-Jun that can be induced by serum and PMA [30–32]. Although activation or overexpression of AP-1 proteins is implicated in the development and progression of many human cancers, distinct roles of AP-1 proteins have also been observed [29,33,34]. For example, reduced expression of c-Fos and c-Jun has been observed in a subset of human prostate cancer patients [35–38], though the clinical significance of reduced AP-1 protein expression remains unclear. Recently, we have demonstrated that c-Jun acts as a transcriptional repressor of the androgen receptor (AR) signaling, and that silencing c-Jun promotes the growth of both androgen-dependent LNCaP cells and castration-resistant C4-2 cells [39], providing evidence that down-regulation of c-Jun expression in a subset of human prostate cancer patients may promote disease progression by enhancing the AR signaling. In the present study, we demonstrate that NF-Y is a major transcription factor to drive PRMT5 transcription in several cancer cell lines, and knockdown of NF-YA leads to down-regulation of PRMT5 expression and suppression of cell growth. Further, we show that PMA treatment in LNCaP cells down-regulates the expression of NF-YA and PRMT5 in a PKC- and c-Fos-dependent manner.

2. Materials and methods

2.1. Cell culture and treatment

The prostate cancer cell lines LNCaP and PC-3 cells were cultured as described previously [40,41]. Lung cancer cell line A549 was kindly provided by Wanqing Liu, and cells were cultured in F-K12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. PMA was purchased from Sigma (P 1585), and bisindolylmaleimide I (GF109203X, GFX), a pan-PKC inhibitor, was purchased from Tocris Bioscience (a gift of the Val Watts lab). For PMA treatment, cells were seeded into 6 cm dishes for 24 h (approximately 80–90% confluence), and then treated with different doses of PMA for the indicated times in the presence or absence of GFX.

2.2. Plasmid construction

Two distinct types of the PRMT5 promoters (−3461/+75 bp and −3474/+75 bp) were amplified from LNCaP cell genomic DNA by PCR with Phusion High-Fidelity DNA Polymerase (NEB) using primers 5′-CGGGGTACCCTGGGCACAACTAGGGCAGAGAAC-3′ and 5′-GAAGAT CTTCCACGCCGGGATTCCTTGATAC-3′. The PCR products were then cloned into pGL4.10 [*luc2*]-Basic Vector (Promega). To construct a series of luciferase reporter genes (A1: −1723/+75, A2: −1156/+75, A3: −459/+75, A4: −323/+75, A5: −240/+75, B1: −1736/+75, B2: −1169/+75, B3: −472/+75, B4: −323/+75, B5: −240/+75, B6: −68/+75, B7: +8/+75), the same methods were used for PCR amplification by using two types of PRMT5 promoters as templates. For mutagenesis, nucleotide substitutions in putative binding motifs were introduced by ligation PCR [42]. The expression plasmids pFLAG-c-Fos and pFLAG-c-Jun were previously constructed [39,43,44]. The cDNA encoding PRMT5 was amplified by PCR using primers 5′-CTGAATTCGG ATGGCGGCGATGGCGGT-3′ and 5′-GCCTCGAGAGAGGCCAATGGTATA TGAGCG-3′ and cloned into pCMV-Myc vector (Clontech). All plasmid constructs were verified with DNA sequencing.

2.3. Luciferase reporter gene assay

Prostate cancer cells were plated in 12-well plates at a density of 2 x 10^5/well, and A549 cells were plated at a density of 1 × 10^5/well. After 24 h, 1 μg of a short-hairpin RNA (shRNA) plasmid targeting NF-YA was transiently co-transfected with 0.5 μg of a PRMT5 reporter gene, along with 0.1 μg of pRL-TK (Promega) by FuGENE HD or FuGENE 6 (Promega). Forty-eight hours after transfection, Firefly and Renilla luciferase activities were determined by a TopCount NXT microplate luminescence counter (Packard) using dual-luciferase Reporter Assay Kit (Promega) according to the manufacturer's instruction with minor modifications as described previously [43,44].

2.4. Immunoblotting

Preparation of total cell lysate (TCL) and immunoblotting were performed as described before [41]. Densitometric quantification was performed with Image J software (NIH, Rockville, MD, USA). The antibodies used for immunoblotting analysis were: anti-β-actin (A1978, Sigma), anti-NF-YA (H-209, sc-10779, Santa Cruz) [45], anti-c-Jun (H-79, sc-1694, Santa Cruz), anti-c-Fos (H125, sc-9202, Santa Cruz), anti-PRMT5 (07-405, Millipore), anti-FLAG M2 (F3165, Sigma), anti-Myc (631206, Clontech), and anti-cyclin A2 (CCNA2, BF683, Cell Signaling). Secondary HRP-conjugated antibodies were purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK).

2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells by using TRIzol reagent (Invitrogen) according to the manufacturer's instruction and verified for integrity by agarose gel electrophoresis. One microgram of RNA was used for reverse-transcription using random primers (100 ng) and MMLV reverse transcriptase (Promega). The mRNA level of PRMT5, NF-YA, NF-YB, NF-YC and GAPDH was quantified using qRT-PCR with gene specific primers. PRMT5 forward, 5′-CAGAGAAGGAGT TCTGCTCCTAC-3′ and PRMT5 reverse, 5′-ATGGCCTGCTGGTACTGAGA GT-3′; NF-YA forward, 5′-CTGTGACACTACCAGTGGCAG-3′ and NF-YA reverse, 5′-TGCCTCCTCTTAAGAATACGG-3′; NF-YB forward, 5′-GCAA GTGAAAGGTGCCATCAAGAG-3′ and NF-YB reverse, 5′-CTGCTCCACCAA TTCCCTTTTCTC-3′; NF-YC forward, 5′-GAACTGAAACCTCCAAAGCGTC-3′ and NF-YC reverse, 5′-TGTGCGATGATGATCTGCCCAG-3′. GAPDH forward, 5′-CTGACTTCAACAGCGACACC-3′ and GAPDH reverse, 5′-CCCTGT TGCTGTAGCCAAAT-3′. qRT-PCR was performed with SYBR@GREEN PCR Master Mix (Roche) by using a ViiA7 Real-Time PCR system (Applied Biosystems) for 40 cycles. The relative expression of each individual gene was normalized to GAPDH and was calculated using the comparative $2^{-\Delta\Delta CT}$ method [46].

2.6. Chromatin immunoprecipitation (ChIP)

Cells cultured in 10 cm dishes were cross-linked with 1% formaldehyde for 10 min and then stopped by adding 125 mM glycine. Chromatin from two-dish cells was sheared by a Branson Digital Sonifier 250 to an average size of approximately 0.5 kb in 1 ml immunoprecipitation (IP) buffer (50 mM Tris·Cl, pH 7.4, 0.5% NP-40, 1% Triton X-100,

150 mM NaCl, 5 mM EDTA, and 0.5 mM DTT). The sheared chromatin (DNA–protein complexes) was incubated with anti-NF-YA (G-2, sc-17753X, Santa Cruz) [47], or the control IgG (sc-2025, Santa Cruz) at 4 °C for overnight and the DNA–protein complexes were recovered by protein G-agarose beads (Santa Cruz, sc-2002). The immunoprecipitated DNA was isolated by 10% Chelex-100 using the fast ChIP method [48], and then subjected to qRT-PCR. The relative fold enrichment was calculated by normalizing to IgG control. A non-target region in the PRMT5 distal promoter and a region containing a validated NF-Y binding site in the CCNA2 promoter were amplified from the same IP sample, and used as negative control and positive control, respectively. The primers used for ChIP are listed as follow: the region containing two NF-Y binding sites in the PRMT5 proximal promoter (5′-CACTGTTTCTCTCCGTGATGGTAC-3′ and 5′-GCGTCTGCCACAGCTCCCGAAC-3′); and a non-target region in the PRMT5 distal promoter (5′-CTGGGCACAACTAGGGCAGAGAAC-3′ and 5′-TTAGTAGAGACGGGGTTTCAC-3′); the region containing one validated NF-Y binding site in the CCNA2 promoter (5′-GCCCCTGCTCAGTT TCCTTTG-3′ and 5′-CGGCGGCTGTTCTTGCAGTTCA-3′).

2.7. Lentivirus production and establishment of stable cell lines

For the construction of shRNA expressing plasmids, the pLKO-Tet-On inducible lentiviral RNAi system was used [49]. Several targeting sequences were selected from the RNAi Consortium (Sigma) as follow: NF-YA (shYA#1), 5′-CCATCGTCTATCAACCAG TTA-3′ (TRCN0000014930); NF-YA (shYA#2), 5′-CCATCATGCAAG TACCTGTTT-3′ (TRCN0000014932); and c-Fos, 5′-GCGGAGACAG ACCAACTAGAA-3′ (TRCN0000273941). Scrambled control (SC), 5′-AACAAGATGAAGAGCACCAA-3′, was used as a negative control for all knockdown experiments. Annealed oligonucleotides were cloned into pLKO-Tet-On. To generate viral particles, HEK 293 T cells were cultured in a 10-cm dish without antibiotics for 24 h, and then co-transfected with 2 μg of pLKO.1-Tet-On shRNA vector, 1.5 μg of pHR'-CMV-ΔR8.2Δvpr packaging plasmid, and 0.5 μg of pHR'-CMV-VSVG envelope plasmid using FuGENE HD reagent. The supernatant containing viruses was harvested 3 days post-transfection, and then filtered through a 0.45 μm filter to remove cell debris. Prostate cancer cells and lung cancer cells were then infected by applying 6 ml viral supernatant in 10 ml complete medium. Polybrene was added to a final concentration of 8 μg/ml to facilitate the infection. Cells were selected with 2 μg/ml of puromycin (for PC-3, 3.5 μg/ml) for 3 days for stable integration of the shRNA plasmids, and surviving cells were maintained in the presence of 1 μg/ml of puromycin. To knock down NF-YA or c-Fos, cells were induced with 1 μg/ml of doxycycline (Dox) for at least 3 days.

2.8. Cell growth analysis and Trypan blue exclusion assay

LNCaP and PC-3, or A549 stable cell lines were seeded in six-well plates in triplicate at a density of 1×10^5 cells/well or 2×10^4 cells/well, respectively. Cells were then induced with or without Dox (1 μg/ml) for various times, and medium and Dox were changed every 3 days during culture. The number of viable and dead cells from each well was determined by Trypan blue staining. To determine the effect of NF-YA knockdown on cell proliferation, the indicated stable cell lines were seeded and grew on coverslips in six-well plates at a cell density of 1×10^5 cells/well or 2×10^4 cells/well, followed by treatment with or without Dox (1 μg/ml) for 84 h. Bromodeoxyuridine (BrdU, Calbiochem Cat#QIA58) was then added to each well for incubation of another 8 h and cells were processed as described previously [39]. For quantification of BrdU-incorporated cells, at least 1000 cells from 10 fields were counted for each cell line under a Nikon TE2000-U inverted fluorescence microscope. Fluorescent images were taken at 200× magnification and the percentage of BrdU positive cells was shown.

2.9. Statistical analyses

Statistical analyses were performed with the GraphPad Prism 6 Software (Graphpad Software, San Diego, CA, USA). Briefly, Student's *t* test was used to compare means of two different groups, while one-way analysis of variance (ANOVA) was used for multiple group comparison, followed by Tukey's post-hoc test or Dunnett's test. Two-way ANOVA was used to compare the means of two independent variables, followed by Tukey's post-hoc test. All data were expressed as mean ± SEM, and *p* values less than 0.05 between groups were considered statistically significant. To analyze the correlation between the expression of PRMT5 and NF-YA in prostate cancer, we searched the Oncomine database (www.oncomine.org) and included each study that has more than 60 samples. A total of six independent studies met this criterion, and the results from these studies were pooled for correlation analysis. For each pair, the statistic Q was calculated to test the homogeneity of effect sizes across studies [50]. It turns out that, for each pair, the effect sizes across studies are not homogeneous (all with p value < 0.0001). Therefore, we employed a random-effects model for the meta-analysis of each pair [51].

3. Results

3.1. Identification of the proximal promoter of PRMT5

To investigate how PRMT5 expression is transcriptionally regulated, we cloned a 3.5kb PRMT5 promoter from LNCaP cells and found that there were two distinct types of promoters that harbor six single nucleotide polymorphisms (SNPs) and one 13 bp insertion/deletion polymorphism (indel) within 1.8 kb (Fig. 1A). To know whether these SNPs may impact the promoter activity, we used the 1.8 kb of the promoter to construct a series of truncated luciferase reporter genes (Fig. 1A). Transfection of these reporter genes into LNCaP cells resulted in at least a 7-fold increase in the promoter activity when compared with the vector control, with the B3 showing the highest activity (Fig. 1B). Similar results were obtained in PC-3 cells (Fig. 1C). However, mutations of all SNPs did not show any significant impact on the reporter gene activity (data not shown). Taken together, these results suggest that these SNPs have negligible effect on the 1.8 kb promoter activity.

To identify a proximal promoter region, we constructed two other reporter genes (B6: −68/+75; B7: +8/+75) (Fig. 1D) and found that further deletions (B6 and B7) dramatically decreased the reporter gene activity in LNCaP cells (Fig. 1D), indicating that the region −240 to +75 is critical for the PRMT5 promoter activity. Similar results were observed in PC-3 cells (Fig. 1D). Since PRMT5 expression is also required for the growth of lung cancer cells (A549) [3], we transfected these reporter genes into A549 cells and observed that the reporter gene activity of B5 in A549 was 2-fold higher than that in LNCaP and PC-3 cells, though a comparable reporter gene activity of B6 and B7 was observed in all three cell lines (Fig. 1D). These results demonstrate that the proximal −240 region is important for PRMT5 transcription in a cell context-dependent manner.

3.2. The two inverted CCAAT boxes are critical for the proximal promoter activity of PRMT5

We next used AliBaba2.1 and TFSEARCH online software to search for putative *cis*-regulatory elements and identified one consensus GATA binding site for GATA binding, one GC box for SP1 binding, and three identical inverted CCAAT boxes for NF-Y binding in the proximal promoter region (Fig. 2A). In order to determine whether these putative binding sites contribute to the proximal promoter activity, we mutated these consensus motifs by site-directed mutagenesis (Fig. 2B), and examined their activities by using the luciferase reporter gene assays. In LNCaP cells, mutation of Y1 or Y2 (from CCAAT to CAGAA) [52], decreased the reporter gene activity by 33% and 21%, respectively

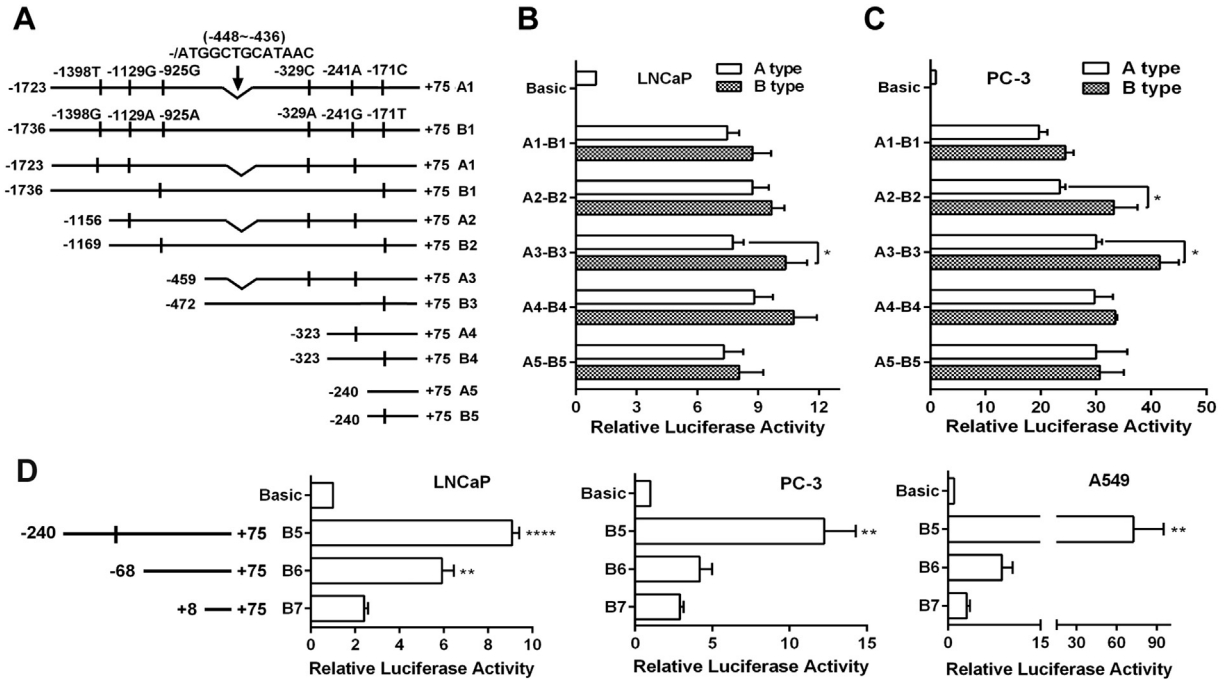

Fig. 1. Identification of the proximal promoter of PRMT5. (A) Two types of PRMT5 promoters cloned from LNCaP genomic DNA with indicated SNPs and an indel, as well as a series of 5′-truncated promoters were used to construct luciferase reporter genes. (B and C) The indicated reporter genes in A were co-transfected with pRL-TK into LNCaP and PC-3 cells for 24 h for measurement of the luciferase activities. Results were obtained from at least three independent experiments in triplicate, and were normalized to the vector control (Basic). (*$p < 0.05$; Student's t test). (D) Luciferase activities of 5′-truncated reporter genes (B6 and B7) in LNCaP, PC-3 and A549 cells. Results from four to six independent experiments are presented as mean ± SEM. Statistical significance (**$p < 0.01$ and ****$p < 0.0001$) was determined when compared with B7 by one-way ANOVA followed by Dunnett's test.

(Fig. 2C). Significantly, mutations of both NF-Y binding sites resulted in 70% reduction in the reporter gene activity. Contrary to the two CCAAT box binding sites, single mutation introduced into the SP1 (GGGCGG to GGAAAG) or GATA (GATA to GCAA) binding site, which was demonstrated previously to abolish their binding [53,54], increased the promoter activity by 36% or 27%, respectively (Fig. 2C). However, mutation of both SP1 and GATA binding sites did not show any further increase in the promoter activity. Similar effect of mutations in NF-Y sites was observed in PC-3 (Fig. 2D) and A549 cells (Fig. 2E), though single mutation of the first NF-Y site (Y1) had a more profound effect compared with the second NF-Y site (Y2). These results suggest that the two NF-Y binding sites may positively regulate PRMT5 transcription in all three cell lines whereas the SP1 and GATA binding sites may negatively regulate PRMT5 transcription in LNCaP cells but not in PC-3 and A549 cells. To know how these binding sites cooperatively contribute to the PRMT5 promoter activity, we mutated these binding sites in combination (Fig. 2B), and observed an overall inhibitory effect on the luciferase reporter gene activity, which was similar to the effect of mutations in the first two NF-Y binding sites (mY1,2). Note that a third NF-Y binding site (Y3) is located at +42, however, mutation of Y3 did not decrease the reporter gene activity in all three cell lines. Instead, a slight increase was observed (Fig. 2F–H). When all three NF-Y binding sites were mutated, a comparable suppression of the reporter gene activity to that with Y1/Y2 mutated was observed in all three cell lines (Fig. 2F–H). Taken together, these results suggest that the first two putative NF-Y binding sites are the major *cis*-regulatory elements to drive PRMT5 transcription.

3.3. NF-Y regulates PRMT5 expression in LNCaP cells via binding to the two CCAAT boxes

Unlike NF-YB and NF-YC, whose expression is relatively stable, NF-YA is the limited subunit for specific binding to CCAAT boxes in cells [12,21–23]. To confirm the role of NF-Y in PRMT5 transcription at the endogenous level, we established two stable cell lines that inducibly express shRNAs targeting two different sequences in the coding region of NF-YA to evaluate the effect of NF-YA knockdown on PRMT5 expression. As shown in Fig. 3A, the two shRNAs knocked down the expression of NF-YA-S, the shorter isoform of NF-YA that is predominantly expressed in LNCaP cells, by more than 65%. The reduction of PRMT5 expression at protein level was similar to that of NF-YA. We confirmed that the expression of a well-known NF-Y target gene CCNA2 was also reduced, demonstrating the specificity of the two NF-YA shRNAs. Since the shYA#1 showed higher knockdown efficiency in LNCaP, it was chosen for the following experiments. We found that knockdown of NF-YA decreased the PRMT5 mRNA level (Fig. 3B), suggesting that the reduction of PRMT5 by NF-YA knockdown likely occurs at the transcriptional level. Transient knockdown of NF-YA significantly inhibited the WT reporter gene activity, but had no effect on the mutant reporter gene activity (Fig. 3C), suggesting that the two CCAAT boxes in the proximal promoter region likely mediates the effect of NF-Y on PRMT5 transcription. We next performed ChIP assays and confirmed that NF-YA bound to the region containing the two CCAAT boxes (P2 in Fig. 3D), but not the distal promoter region that does not contain CCAAT box (P1 in Fig. 3D). As a positive control, NF-Y also bound to the proximal promoter of CCNA2 [55]. These results demonstrate that NF-Y indeed binds to the two CCAAT boxes in the proximal promoter of PRMT5 and regulates PRMT5 transcription in LNCaP cells. To know whether NF-Y may regulate PRMT5 expression in human prostate cancer tissues, we searched Oncomine database and found that there was a strong positive correlation between the transcript level of NF-YA and PRMT5 (Fig. 3E), as evidenced by a meta-analysis from six independent studies. This result further supports our finding that NF-Y regulates PRMT5 expression in prostate cancer cells.

3.4. NF-Y regulation of PRMT5 expression is required for prostate cancer cell growth

Given that NF-Y is critical for PRMT5 expression in several cancer cell lines, we next sought to determine the importance of NF-Y regulation of

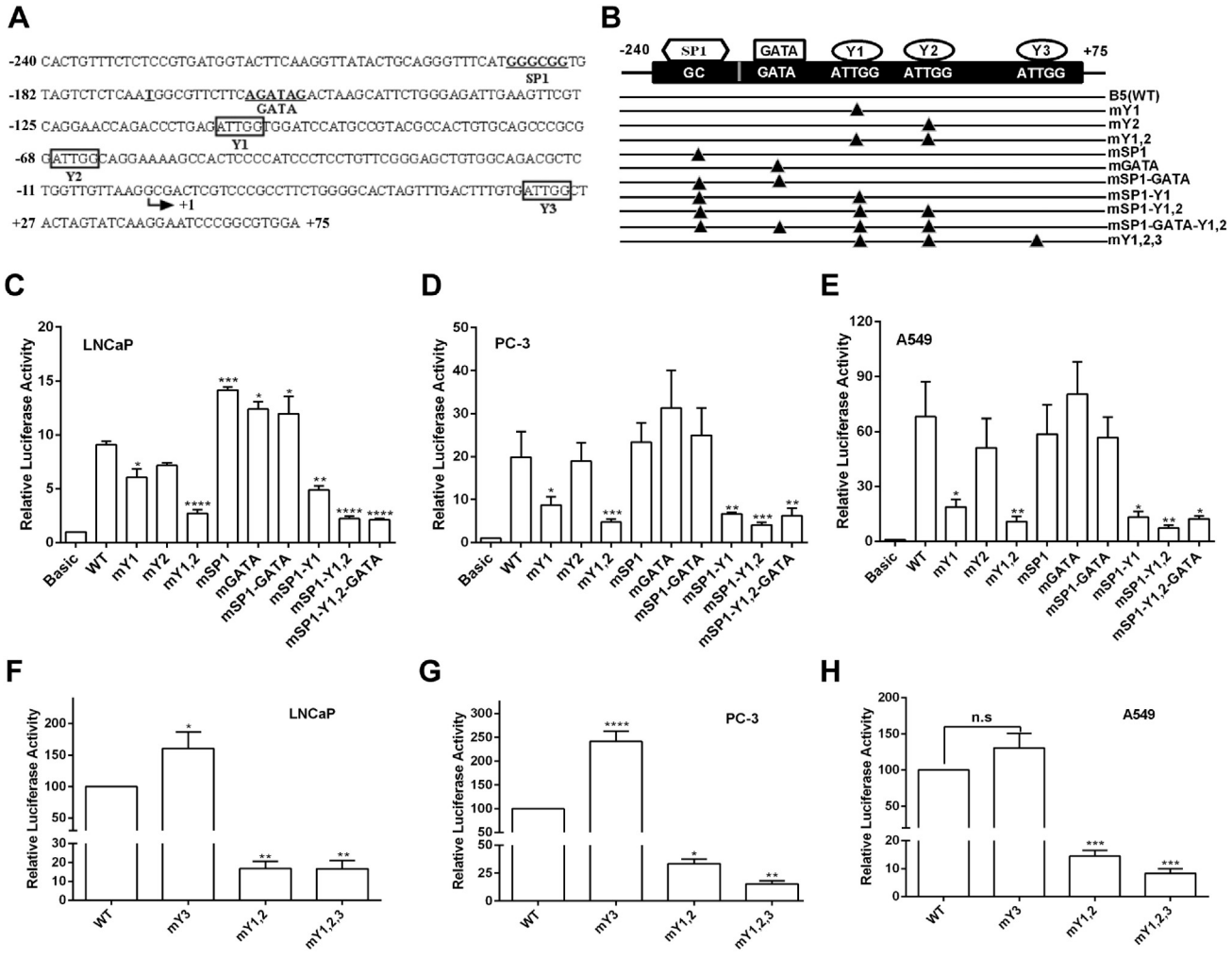

Fig. 2. The two CCAAT boxes are critical for the proximal promoter activity of PRMT5. (A) Sequences of the proximal promoter region from −240 to +75 with predicted *cis*-regulatory elements. The transcription start site was indicated by arrow. Y1, Y2, or Y3 indicates the first, second or third NF-Y binding site. (B) Illustration of a series of B5-based luciferase reporter gene constructs. Triangle indicates the corresponding *cis*-regulatory element was mutated. (C–E) CCAAT boxes are critical for luciferase activity driven by the PRMT5 promoter. The luciferase activity of the indicated reporter gene constructs in B was determined in the indicated cancer cell lines. (F–H) The third NF-Y binding site has little effect on the PRMT5 promoter activity. The indicated luciferase reporter gene was co-transfected with pRL-TK into LNCaP (F), PC-3 (G) and A549 (H) cells for 24 h, and the relative luciferase activity was determined. Results in C–H were from at least three independent experiments, and were normalized to the vector control and are presented as mean ± SEM. Statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$) was determined when compared with WT (wild-type) by one-way ANOVA followed by Dunnett's test.

PRMT5 expression in cell growth. Using the two shRNA constructs, we were able to establish a stable cell line by using A549 to knockdown NF-YA by 50%, accompanied by a 39% reduction in PRMT5 expression (Supplementary Fig. S1A). However, the two shRNAs did not exhibit acceptable knockdown efficiency in PC-3 (Supplementary Fig. S1B). We then examined the effect of NF-YA knockdown on cell growth and cell death in LNCaP and A549. Knockdown of NF-YA inhibited cell growth in LNCaP and A549 cells (Fig. 4A and B). The inhibition of cell growth in both LNCaP and A549 by NF-YA knockdown was attributable to the inhibition of cell proliferation (Fig. 4C and D; Supplementary Fig. S1C and D) and the induction of cell death (Fig. 4E and F), in agreement with previous findings that NF-Y plays a role in regulating cell proliferation and cell death [12]. Because NF-Y may influence growth of these cancer cells by controlling expression of many other genes [12,15,17], we next performed a PRMT5 rescue experiment to determine to what extent PRMT5 down-regulation is responsible for cell growth inhibition induced by NF-YA knockdown. As shown in Fig. 4G and H, transient expression of PRMT5 partially rescued cell growth inhibition only in LNCaP cells, but not in A549 cells. Taken together, these results suggest that the regulation of cell growth by NF-Y may be partially mediated through up-regulation of PRMT5 expression in a cell context-dependent manner.

3.5. The PKC signaling negatively regulates PRMT5 expression in LNCaP cells

We next searched for possible cell signaling that may regulate PRMT5 expression in LNCaP cells by treating cells with various protein kinase inhibitors or agents that activate cell signaling pathways, and observed that treatment of cells with PMA resulted in a dramatic decrease of PRMT5 expression in a dose- and time-dependent manner (Fig. 5A and B). Interestingly, NF-YA expression was similarly inhibited (Fig. 5A and B). Significantly, the mRNA level of PRMT5 (Fig. 5C) and NF-YA, but not NF-YB and NF-YC (Fig. 5D), was inhibited by PMA treatment as well. Because PMA-induced PKC activation contributes to cell growth inhibition and apoptosis in LNCaP cells [26], we examined whether inhibition of PKC can restore the expression of NF-YA and PRMT5 in LNCaP cells, and found that treatment of cells with a pan-PKC inhibitor GFX completely restored the expression of NF-YA and PRMT5 at mRNA and protein level (Fig. 5C–E). The observed increase in NF-YB mRNA in cells treated with PMA plus GFX was likely due to the effect of GFX alone, because GFX treatment only increased NF-YB expression at the mRNA level but had no effect on the expression of PRMT5, NF-YA, and NF-YC (Supplementary Fig. S2). Consistent with a role for NF-Y in regulating PRMT5 transcription via the NF-Y binding sites in the proximal promoter region, PMA treatment resulted in almost

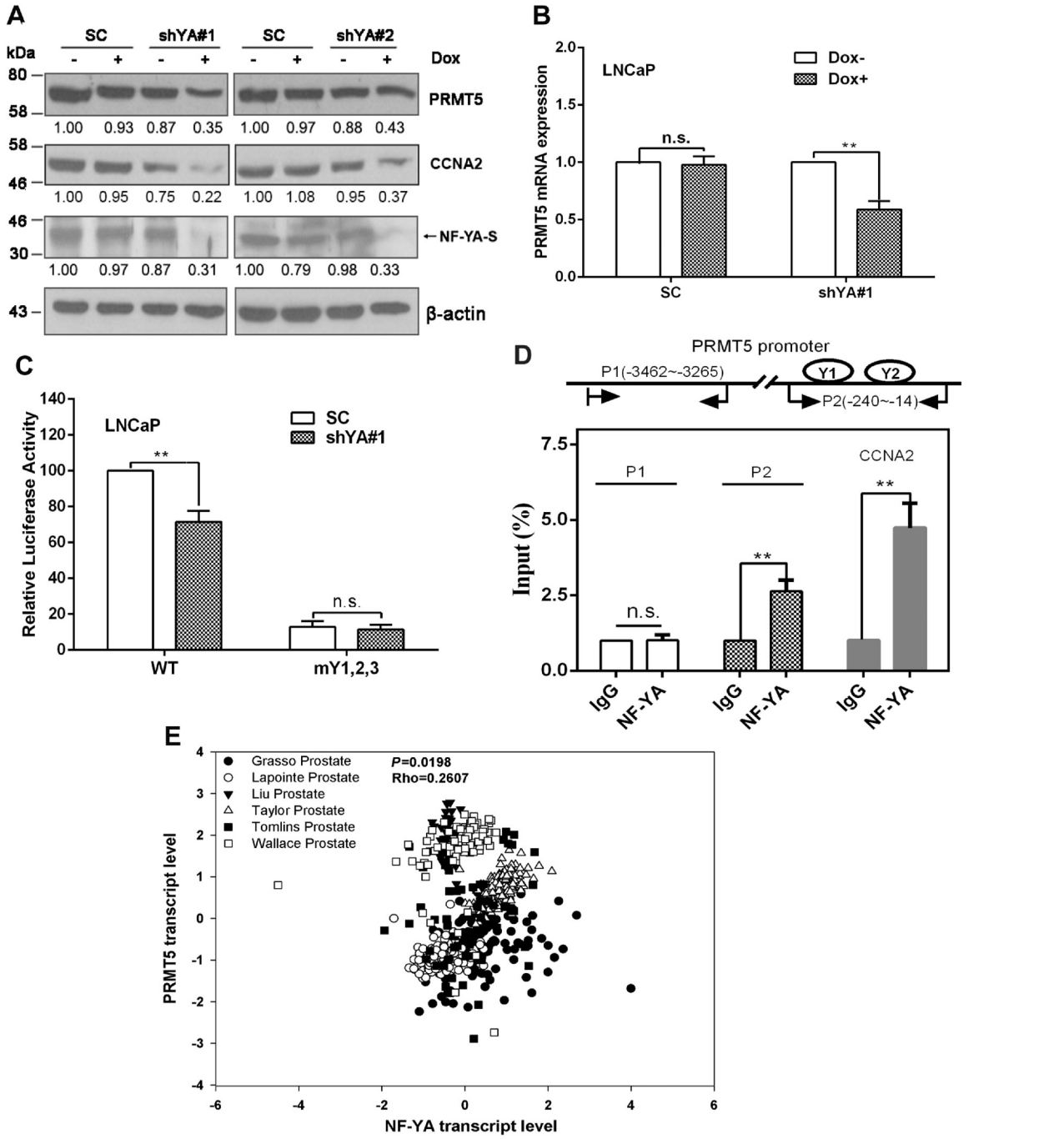

Fig. 3. NF-Y is essential for PRMT5 expression in LNCaP cells. (A) NF-YA knockdown inhibits PRMT5 expression. Doxycycline (Dox) was added at 1 μg/ml for 96 h to induce NF-YA knockdown in ShYA#1 and shYA#2 stable cell lines, and total cell lysate was used for immunoblotting analysis of PRMT5, shorter isoform of NF-YA (NF-YA-S), CCNA2 and β-actin. Shown are representative blots from three independent experiments, and the numbers indicate relative fold changes analyzed by Image J. (B) Knockdown of NF-YA inhibits PRMT5 mRNA expression. shRNA expression was induced by Dox for 72 h, and qRT-PCR was performed to determine the mRNA level of PRMT5. Results are mean ± SEM from four independent experiments, and Student's *t* test was used for statistical analysis (**$p < 0.01$). (C) Knockdown of NF-YA decreases the PRMT5 proximal promoter activity. One microgram of plasmids encoding SC or shYA1# (with Dox induction) was co-transfected with 0.5 μg of the B5 reporter gene plasmid (WT) or the mutant reporter gene (mY1,2,3), along with 100 ng of pRL-TK into LNCaP for 48 h, and dual-luciferase reporter assays were performed and analyzed. Luciferase activities are presented as percentage from at least three independent experiments. **$p < 0.01$. (D) NF-YA binds to the two inverted CCAAT boxes. Shown (top) is a schematic of the two regions (P1 and P2) in the PRMT5 promoter for ChIP analysis. Results (bottom) are mean ± SEM from four independent experiments (**$p < 0.01$). The binding of NF-YA to the CCNA2 promoter was used as a positive control. (E) The transcript level of NF-YA positively correlates with the transcript level of PRMT5 in prostate cancer. Data shown are a meta-analysis from six independent studies deposited in Oncomine database.

75% reduction of the NF-YA binding to the proximal promoter region of PRMT5 (Fig. 5F). In agreement with previous findings that PMA inhibits cell growth and induces apoptosis only in LNCaP, but not in DU 145 and PC-3 cells [25,26], PMA treatment did not cause any significant change in NF-YA and PRMT5 expression in PC-3 cells (Fig. 5G). Additionally, PMA did not have any effect on NF-YA and PRMT5 expression in A549 cells (Fig. 5H). Thus, PMA treatment appears to have a specific effect on the expression of NF-YA and PRMT5 in LNCaP cells.

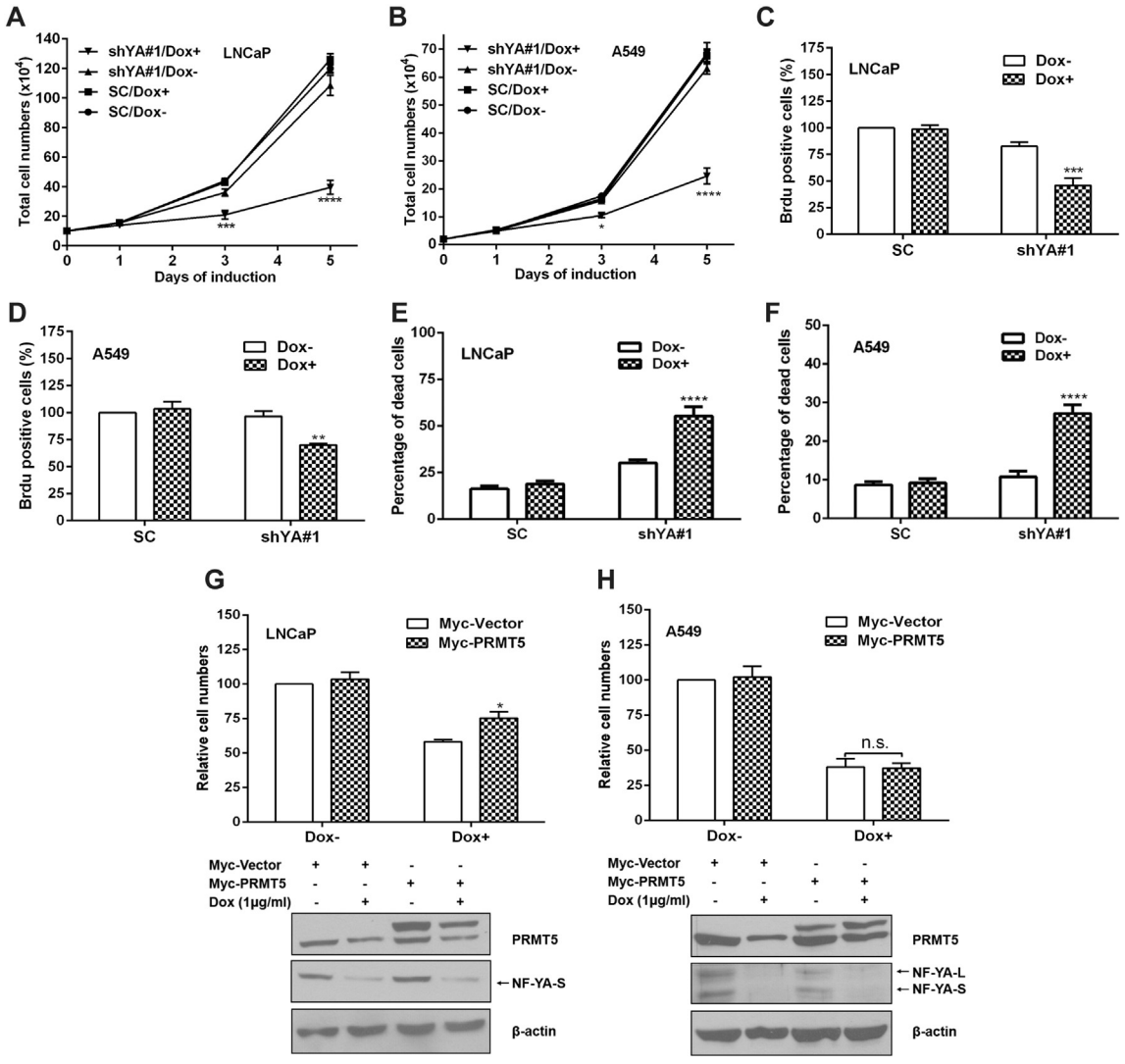

Fig. 4. NF-Y regulation of PRMT5 expression is required for prostate cancer cell growth. (A and B) Knockdown of NF-YA inhibits cell growth in LNCaP (A) and A549 (B). Stable cell lines SC and shYA#1 were induced with 1 μg/ml of doxycycline (Dox+) to express shRNAs or without treatment (Dox−) for the indicated times, and cell numbers were counted using hemocytometer. Results from four independent experiments in duplicate are presented as mean ± SEM. Statistical significance (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$) was determined by two-way ANOVA followed by Tukey's test. (C and D) Knockdown of NF-YA decreases BrdU-incorporated positive cells in LNCaP and A549 cells. SC and shYA#1 stable cell lines were induced with 1 μg/ml of Dox (Dox+) or without treatment (Dox−) for 84 h, followed by BrdU treatment for another 8 h. Number of BrdU-positive cells was determined using Image J software (total cell number > 1000, n = 10). Results obtained from four independent experiments in duplicate are presented as mean ± SEM. Statistical significance (**$p < 0.01$; ***$p < 0.001$) when compared with SC was determined by two-way ANOVA followed by Dunnett's test. (E and F) Effect of NF-YA knockdown on cell death. Stable and inducible cell lines targeting NF-YA (shYA#1) or the SC control were cultured in 6 cm dishes, and induced with 1 μg/ml of Dox (Dox+) or without treatment (Dox−) for 72 h. Cells were trypsinized and counted to determine the percentage of dead cells by Trypan blue exclusion method. (G and H) Overexpression of PRMT5 rescues cell growth inhibition induced by NF-YA knockdown in LNCaP cells, but not in A549. LNCaP and A549 stable cell lines expressing shYA#1 were induced with 1 μg/ml of Dox (Dox+) or without induction (Dox−) for 48 h, followed by transient transfection with pCMV-Myc (Myc-vector) or pCMV-Myc-PRMT5 (Myc-PRMT5) and incubation for another 48 h. Top, results are presented as mean ± SEM from three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's test. *$p < 0.05$; n.s., no significance. Bottom, the expression level of PRMT5 and NF-YA was determined by immunoblotting analysis. Shown are representative blots from three independent experiments. Note that the expression of both NF-YA longer isoform (NF-YA-L) and shorter isoform (NF-YA-S) was detectable in A549 cells whereas the expression of NF-YA-S was detectable in LNCaP cells only.

3.6. c-Fos mediates the PKC signaling to regulate PRMT5 transcription via down-regulation of NF-YA expression

As AP-1 proteins c-Fos and c-Jun are downstream transcription factors of PKC that can be induced by PMA [30–32], we confirmed that PMA treatment indeed induced expression of c-Fos and c-Jun in LNCaP cells (Fig. 6A). However, overexpression of c-Fos, but not c-Jun, inhibited the PRMT5 reporter gene activity (Fig. 6B). Consistent with its effect on the PRMT5 reporter gene activity, overexpressed c-Fos, but not c-Jun, decreased PRMT5 mRNA (Fig. 6C) and protein expression (Fig. 6D). We found that NF-YA expression at both mRNA and protein levels was also inhibited by c-Fos (Fig. 6C and D). These results suggest that c-Fos may mediate the PKC signaling to down-regulate the expression of NF-YA and PRMT5. To test this, we generated a shRNA construct targeting c-Fos and observed that knockdown of c-Fos increased the PRMT5 reporter gene activity by 54% (Fig. 6E). Further, we used the shRNA construct to establish an inducible stable cell line to knock down c-Fos, and observed that PMA-induced NF-YA and PRMT5 down-regulation was partially restored when c-Fos was knocked down (Fig. 6F and G). Since the ENCODE ChIP-seq data from the UCSC database (http://genome.ucsc.edu/ENCODE/) show that c-Fos also binds to the proximal promoter region in HeLa-S3 and K562 cells, we were interesting to know whether c-Fos has any direct impact on the PRMT5 promoter activity in LNCaP cells. To this end, we examined the effect of c-Fos overexpression or knockdown on the WT and the mutant PRMT5 reporter gene activity. As shown in Fig. 6H and I, we found that

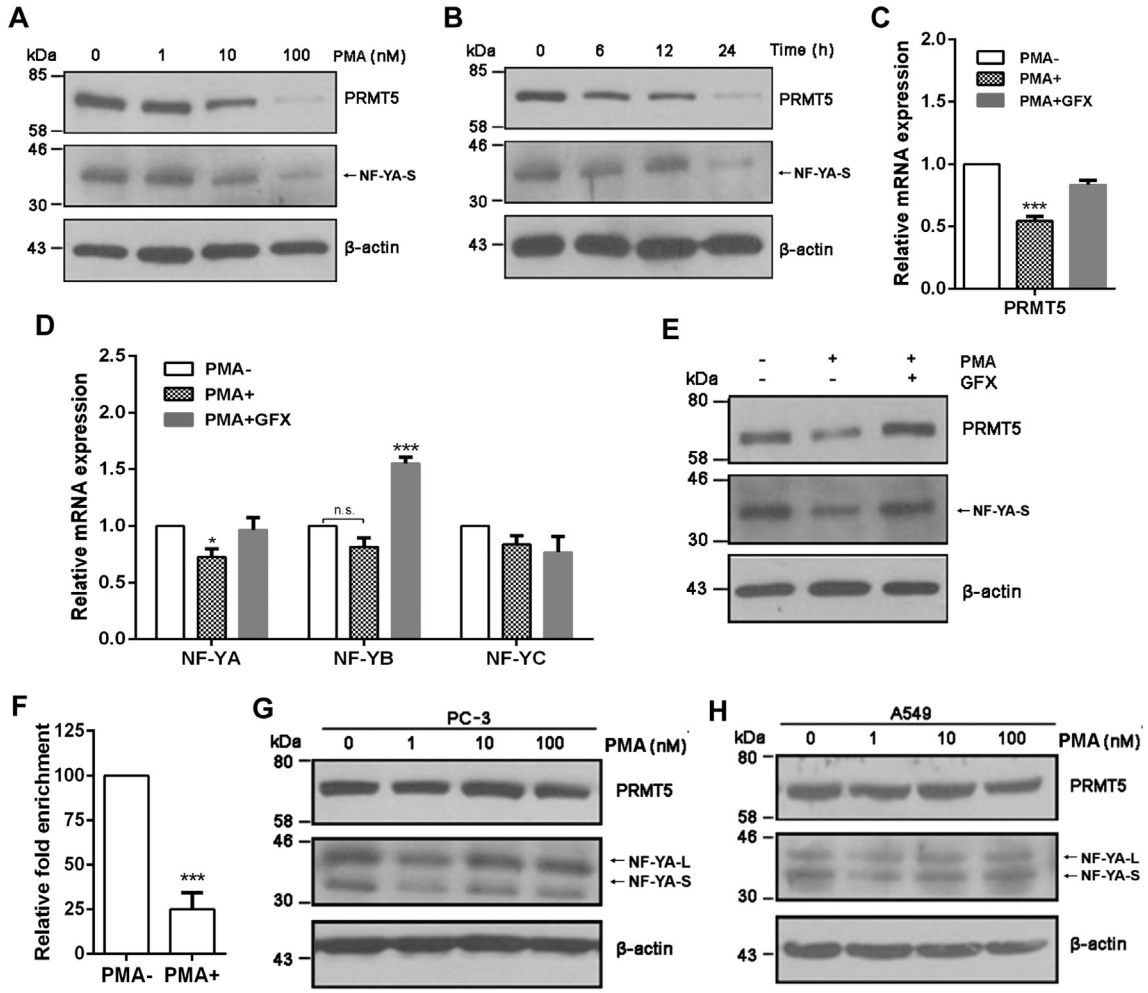

Fig. 5. PKC negatively regulates PRMT5 expression in LNCaP. (A and B) The PKC activator PMA inhibits NF-YA and PRMT5 expression in a dose- and time-dependent manner. LNCaP cells were treated with PMA at the indicated doses (A) for 24 h or treated with 100 nM of PMA for the indicated time points (B), and total cell lysate was used for immunoblotting analysis of PRMT5 and NF-YA expression. (C and D) A pan-PKC inhibitor inhibits PMA-induced down-regulation of PRMT5 and NF-YA at the mRNA level. LNCaP cells were treated with 100 nM of PMA in the presence or absence of a pan-PKC inhibitor GFX (200 nM) for 24 h, and relative mRNA level of PRMT5 (C) or NF-YA, NF-YB and NF-YC (D) was determined by qRT-PCR. Results from three independent experiments are presented as mean ± SEM in C and D, and statistical significance ($^{*}p < 0.05$, $^{***}p < 0.001$) was determined by one-way ANOVA followed by Tukey's test. (E) PKC inhibition restores NF-YA and PRMT5 expression at the protein level in cells treated with PMA. LNCaP cells were treated with 100 nM of PMA in the presence or absence of GFX (200 nM) for 24 h, then NF-YA and PRMT5 expression was analyzed by immunoblotting. Representative blots from three independent experiments are shown. (F) PMA treatment decreases NF-YA binding to the PRMT5 promoter. ChIP analysis was conducted using anti-NF-YA antibody to determine the binding of NF-YA to the two CCAAT boxes in the proximal promoter region of PRMT5. $^{***}p < 0.001$ (Student's *t* test). (G and H) PMA does not significantly affect the expression of NF-YA and PRMT5 in PC-3 and A549. PC-3 and A549 cells were treated with PMA at the indicated concentration for 24 h, and total cell lysate was used for immunoblotting detection of NF-YA and PRMT5 expression. PMA −, DMSO treatment (Fig. 5C–F).

overexpression of c-Fos decreased the WT PRMT5 reporter gene activity by 62.3%, but had no effect on the mutant reporter gene activity in which all three NF-Y binding sites were mutated (mY1,2,3). In contrast, transient knockdown of c-Fos remarkably increased the WT PRMT5 reporter gene activity, but had no effect on the mutant reporter gene activity. These results provide evidence that c-Fos indeed mediates, at least partially, the PKC signaling to negatively regulate PRMT5 transcription via down-regulation of NF-YA in LNCaP cells.

4. Discussion

It has been reported that PRMT5 may function as an oncogene to promote cancer cell growth [1–3,5–7,9,10]. Although NF-Y directly regulates transcription of many target genes to control cell cycle progression, cell proliferation and cell survival [12,13,15,17], our finding that NF-Y transcriptionally activates PRMT5 expression suggests that NF-Y may also regulate cancer cell growth by controlling the expression level of PRMT5, an emerging epigenetic enzyme that functions as an oncogene in human cancers [1]. For example, E2F1 is a member of the E2F family transcription factor required for transactivation of target genes involved in cell cycle progression in cancer cells [56]. Because the transcriptional activity of E2F1 is under the control of the tumor suppressor Rb, loss of Rb leads to constitutive activation of E2F1 and cancer development [57]. Interestingly, PRMT5 can epigenetically silence transcription of Rb [9]. Thus, activation or overexpression of NF-Y may lead to PRMT5 overexpression, by which Rb is silenced and E2F1 is activated, providing another pathway to promote cell cycle progression in cancer cells that harbor the wild-type Rb gene [9]. As NF-Y also regulates the transcription of the same target genes such as E2F1 [58], future studies of how NF-Y coordinates the regulation of PRMT5 expression and other target genes will likely provide novel insights into the oncogenic role of both NF-Y and PRMT5 in cancer cells.

Recent evidence indicates that PRMT5 is overexpressed in multiple human cancers [3–11], though it is unknown how PRMT5 expression is regulated by cancer signaling. In leukemia and lymphoma cells, down-regulation of several miRNAs contributes to PRMT5 overexpression [9,10]. We have provided several lines of evidence that NF-Y regulates PRMT5 transcription via the binding to the two CCAAT boxes in the proximal promoter region of PRMT5. First, mutagenesis analyses showed that mutation of the two CCAAT boxes in the proximal

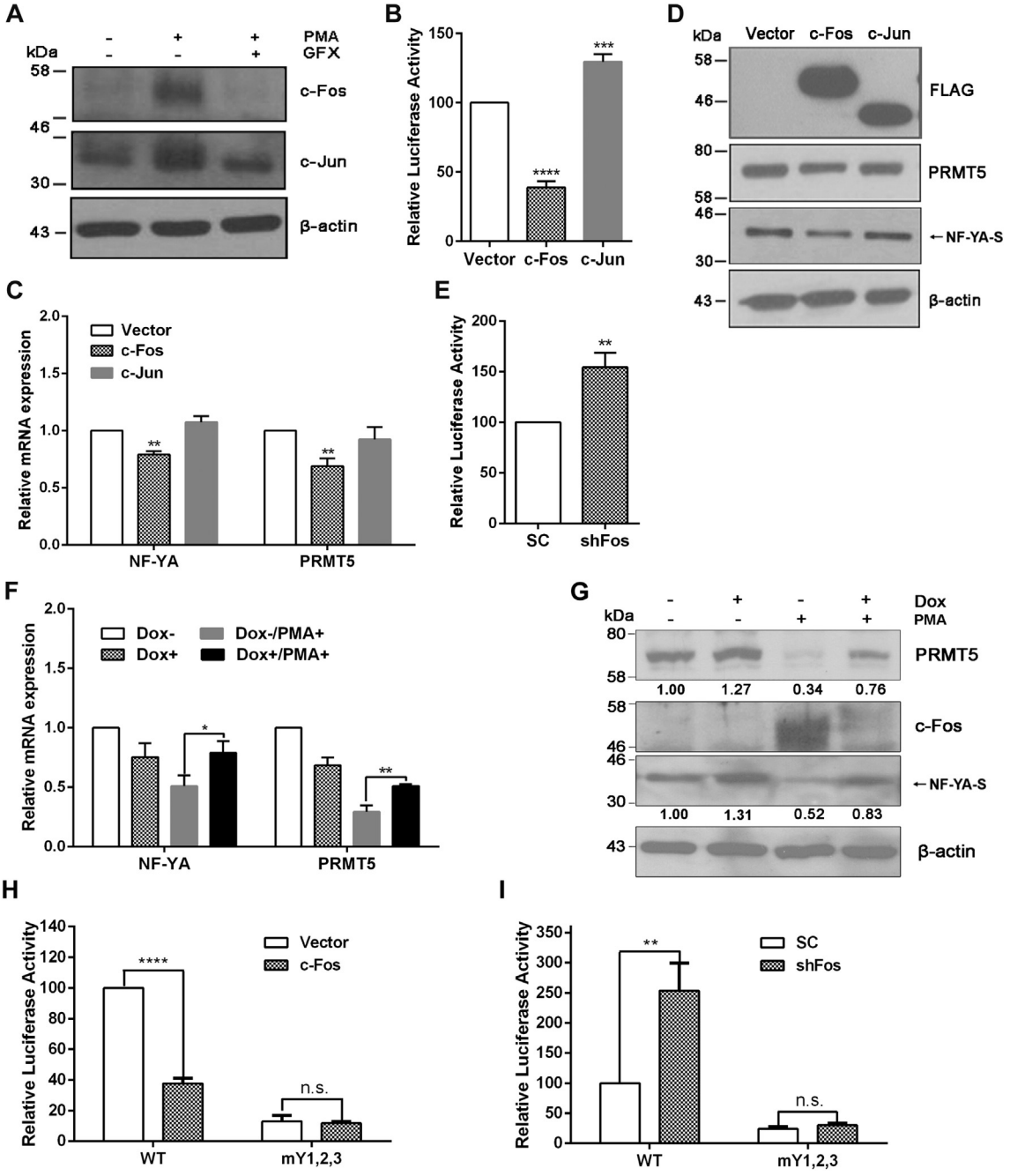

Fig. 6. c-Fos mediates the PKC signaling to down-regulate PRMT5 expression via NF-YA. (A) PMA increases c-Jun and c-Fos expression in LNCaP. LNCaP cells were treated with 100 nM of PMA in the presence or absence of GFX (200 nM) for 24 h, and the expression of c-Fos and c-Jun was determined by immunoblotting. (B) Overexpression of c-Fos, but not c-Jun, inhibits the PRMT5 promoter activity. One microgram of pCMV-FLAG (Vector), pFLAG-c-Fos (c-Fos) or pFLAG-c-Jun (c-Jun) was co-transfected with 0.5 μg of the wild-type (B5) reporter gene, along with 0.1 μg of pRL-TK into LNCaP cells. The luciferase activity was determined 24 h after the transfection. Results from six independent experiments in triplicate are presented as mean ± SEM, and statistical significance (***$p < 0.001$, ****$p < 0.0001$) was determined using one-way ANOVA followed by Dunnett's test. (C and D) Overexpression of c-Fos, but not c-Jun, inhibits NF-YA and PRMT5 expression. LNCaP cells were transfected with 3 μg of the indicated plasmids as described in B. The mRNA and protein expression of NF-YA and PRMT5 was determined by qRT-PCR (C) and immunobloting (D), respectively. Results from at least three independent experiments are presented as mean ± SEM. Statistical significance (**$p < 0.01$) was determined by using one-way ANOVA followed by Dunnett's test. (E) Knockdown of c-Fos increases the PRMT5 promoter activity. The SC or c-Fos shRNA (shFos) was co-transfected with 0.5 μg of the wild-type (B5) reporter gene, along with 0.1 μg of pRL-TK into LNCaP cells. The luciferase activity was determined 48 h after the transfection. **$p < 0.01$ versus SC (Student's t test). (F and G) Knockdown of c-Fos partially rescues NF-YA and PRMT5 expression. Stable cell line that can inducibly express a c-Fos shRNA was induced with 1 μg/ml of doxycycline (Dox +) or without treatment (Dox −) for 48 h. Cells then were treated with 100 nM of PMA (PMA +) or DMSO (PMA −) for another 24 h, followed by determination of the mRNA expression (F) and protein expression (G) of NF-YA and PRMT5. Statistical significance (*$p < 0.05$, **$p < 0.01$) was determined by two-way ANOVA followed by Tukey's test. The numbers in G indicate the relative expression level of each protein analyzed by Image J software. (H and I) c-Fos decreases PRMT5 promoter activity mainly through CCAAT boxes. The indicated plasmids were transfected into LNCaP cells, and the luciferase assays were performed following the same procedure as described in B and E, respectively. Results from three independent experiments in triplicate are presented as mean ± SEM, and statistical significance (**$p < 0.01$, ****$p < 0.0001$).

promoter region resulted in 70% reduction in the luciferase reporter gene activity in three different cancer cell lines (Fig. 2C–E). Second, endogenous NF-YA also specifically bound to the proximal promoter region containing the two CCAAT boxes in LNCaP cells (Fig. 3D). Third, knockdown of NF-YA not only inhibited the PRMT5 promoter-driven luciferase report gene activity but also decreased the expression of PRMT5 at both mRNA and protein levels (Fig. 3A–C). We also show that the PKC/c-Fos signaling negatively regulates PRMT5 expression

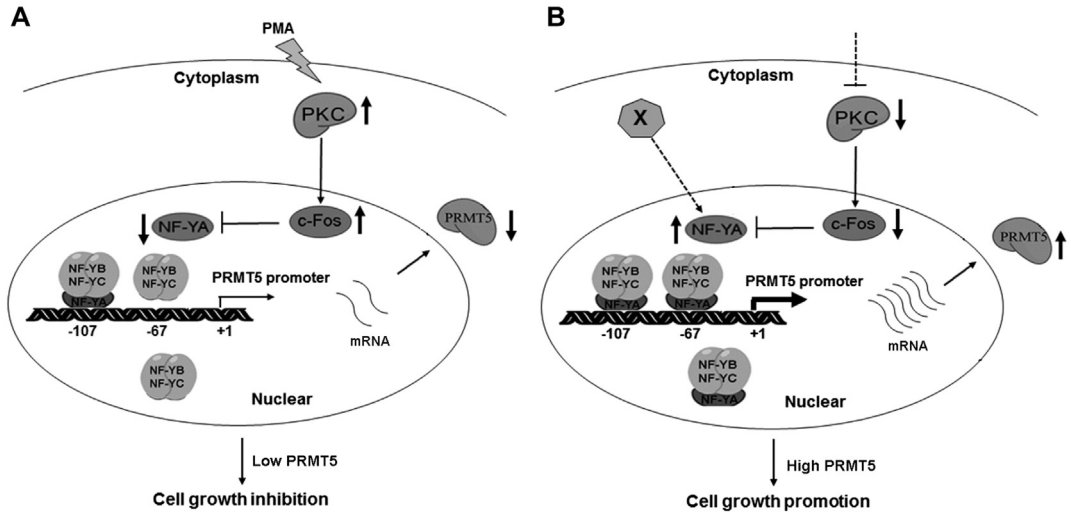

Fig. 7. Model for the regulation of PRMT5 expression by the PKC-c-Fos-NF-Y signaling in human cancer. (A) The PKC signaling negatively regulates PRMT5 expression in a c-Fos- and NF-Y-dependent manner in LNCaP cells. In response to PMA treatment, activation of PKC leads to the induction of c-Fos, which in turn suppresses NF-YA transcription and results in down-regulation of PRMT5. As a result, cell growth is inhibited. (B) Proposed mechanisms underlying up-regulation of PRMT5 expression in cancer cells. Two possible mechanisms may underlie PRMT5 overexpression in human cancers. One is the inactivation or down-regulation of PKC by cell signaling, and the other is direct activation or up-regulation of NF-YA by cell signaling that remains to be identified (X). Dashed lines indicate unknown factors that remain to be identified. Thick solid arrows illustrate the up-regulation or down-regulation of the indicated protein.

via down-regulation of NF-YA transcription in LNCaP prostate cancer cells (Figs. 5–7A). Although the mechanism by which c-Fos represses NF-YA transcription remains to be investigated, it is interesting to note that our preliminary analysis of the NF-Y promoter identified three consensus AP-1 binding sites within the 6 kb promoter region. It is therefore possible that c-Fos may directly repress NF-YA transcription by binding to these consensus AP-1 binding sites. Alternatively, c-Fos may indirectly repress NF-YA transcription through a secondary effect (e.g., up-regulation of a transcriptional repressor of NF-YA). Nevertheless, our findings suggest that cell signaling may up-regulate PRMT5 expression by down-regulation of PKC or by direct up-regulation of NF-YA to promote cancer cell growth (Fig. 7B). This is further supported by the fact that several isozymes of PKC are down-regulated in human cancers [59]. Indeed, a preliminary analysis of the Oncomine database shows that the transcript level of several PKC isozymes inversely correlates with the transcript level of PRMT5 in prostate cancer and lung cancer (Supplementary Fig. S3). It will be interesting to see whether down-regulation of these PKC isozymes correlates with PRMT5 overexpression at the protein level in human cancer tissues.

The cell growth-promoting role of PRMT5 is mediated by controlling the expression of target genes or by post-translational modification of signaling molecules that are involved in cell cycle progression, apoptosis and DNA repair [1]. Although knockdown of PRMT5 in LNCaP cells inhibits cell proliferation [60], the downstream signaling mediating this effect remains unknown. A previous study suggests that PRMT5 may be required for the transcriptional activity of AR in a luciferase reporter gene assay [61]. Given that PMA-induced down-regulation of PRMT5 is mainly observed in AR positive LNCaP cells, but not in AR negative DU 145 and PC-3 cells, it is plausible to hypothesize that down-regulation of PRMT5 by PMA in LNCaP cells may contribute to the suppression of LNCaP cell growth and induction of apoptosis by attenuating the AR activity [61]. As a recent report shows that PMA treatment in LNCaP cells can down-regulate AR expression [62], it would be interesting to examine whether PRMT5 has any effect on AR expression. Alternatively, PMA-induced PRMT5 down-regulation may contribute to PMA-induced apoptosis by enhancing the activity of p38δ, a major serine/threonine protein kinase mediating PMA-induced apoptosis in LNCaP cells [26]. Support for this notion comes from a recent observation that PRMT5 forms a complex with p38δ and suppresses PKCδ- and p38δ-dependent signaling in keratinocytes [63]. Future studies to distinguish these possibilities will provide a novel insight into the regulatory role of PRMT5 in prostate cancer cells.

In summary, we have identified NF-Y as the major transcriptional activator of PRMT5 in multiple cancer cell lines, and demonstrated that the PKC/c-Fos signaling negatively regulates PRMT5 expression in LNCaP prostate cancer cells through down-regulation of NF-YA transcription. Because down-regulation of several PKC isozymes correlates with human cancer development and progression [59], further analysis of the interplay between PRMT5 and the PKC/c-Fos signaling in human cancer will provide novel insights into the oncogenic role of RPMT5 in human cancers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2014.09.015.

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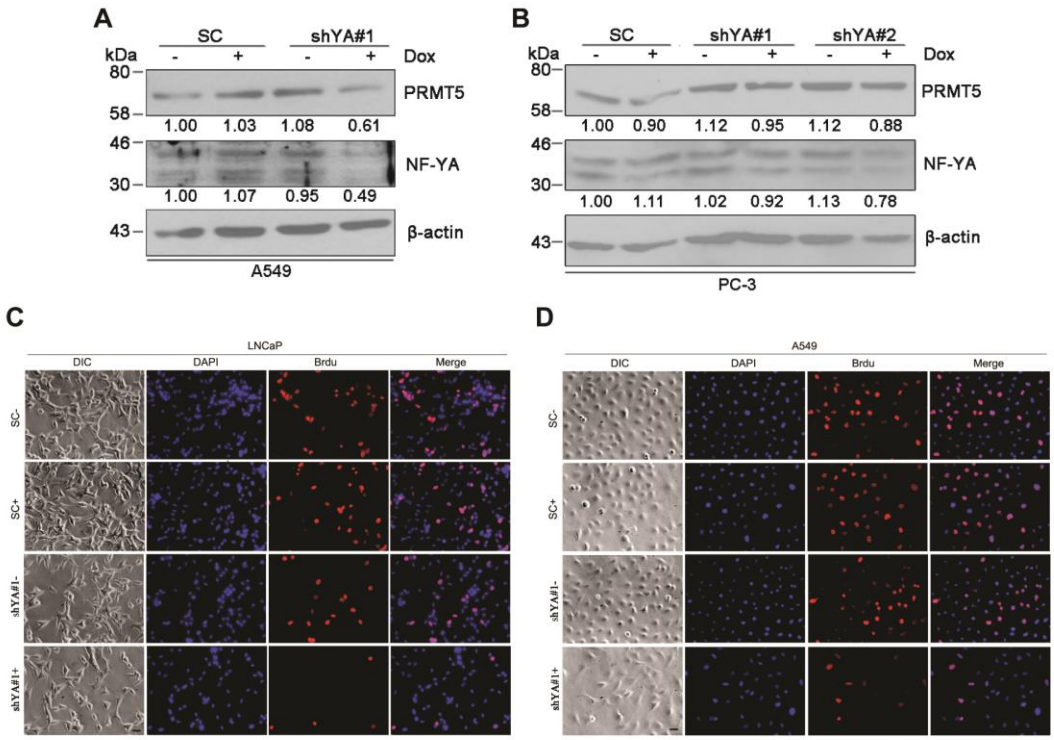

Fig. S1. Effect of PRMT5 knockdown on cell proliferation in LNCaP and A549 cells. (A) Knockdown of NF-YA decreases PRMT5 expression in A549 cells. A549 stable cell lines expressing shYA#1 or the scrambled control (SC) were induced to knock down NF-YA by 1 μg/ml of doxycycline (Dox) for 96 hours. Immunoblotting was applied to analyze expression of NF-YA and PRMT5. The number of values indicates the relative expression determined by Image J. (B) PC-3 stable cell lines expressing shYA#1 or shYA#2 or the scrambled control (SC) were induced to knock down NF-YA by 1 μg/ml of doxycycline (Dox) for 96 hours. Results were analyzed as in (A). (C and D) Knockdown of NF-YA inhibits Brdu incorporation in LNCaP and A549 cells. LNCaP and A549 stable cell lines were induced with and without Dox (1 μg/ml) for 84 hours, followed by BrdU treatment for another 8 hours. Cells were fixed and immunostained with a BrdU-specific antibody (Red). The nucleus was stained with DAPI (Blue). Scale bar: 50 μm.

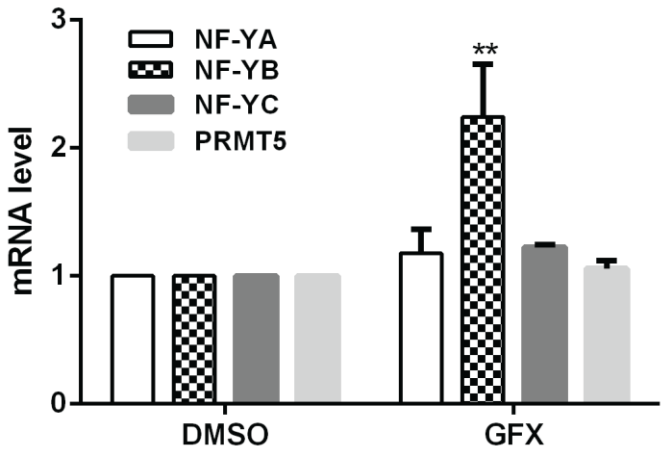

Fig. S2. Effect of GFX on mRNA expression of PRMT5 and NF-Y subunits. LNCaP cells were treated with GFX (200 nM) or DMSO for 24 hours. The mRNA expression of NF-YA, NF-YB, NF-YC and PRMT5 was determined by qPCR. Student's *t* test was used for statistical analysis (**, $p<0.01$).

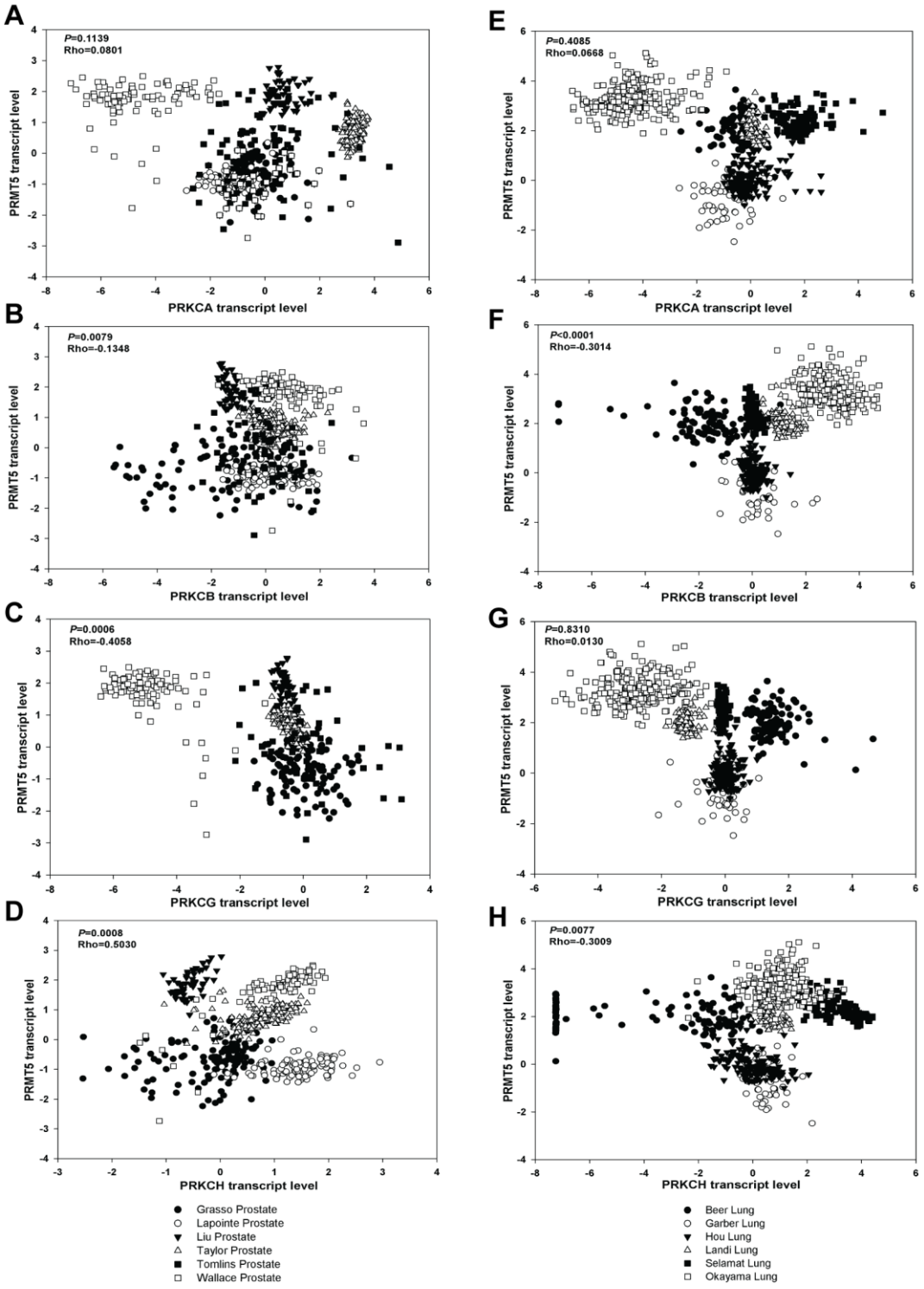

Fig. S3. The correlation between PKC isozymes and PRMT5 transcript in cancer tissues. Expression of several PKC isozymes correlates with expression of PRMT5 in prostate cancer (A-D) and lung cancer (E-H). Data shown are from six independent studies (each study has more than 60 samples) deposited in Oncomine database (www.oncomine.org). All these studies were pooled for correlation analysis, and a random-effects model was employed for the meta-analysis of each pair.

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The E3 ubiquitin ligase CHIP mediates ubiquitination and proteasomal degradation of PRMT5

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ABSTRACT

Protein arginine methyltransferase 5 (PRMT5) is an important member of the protein arginine methyltransferase family that regulates many cellular processes through epigenetic control of target gene expression. Because of its overexpression in a number of human cancers and its essential role in cell proliferation, transformation, and cell cycle progression, PRMT5 has been recently proposed to function as an oncoprotein in cancer cells. However, how its expression is regulated in cancer cells remains largely unknown. We have previously demonstrated that the transcription of PRMT5 can be negatively regulated by the PKC/c-Fos signaling pathway through modulating the transcription factor NF-Y in prostate cancer cells. In the present study, we demonstrated that PRMT5 undergoes polyubiquitination, possibly through multiple lysine residues. We also identified carboxyl terminus of heat shock cognate 70-interacting protein (CHIP), an important chaperone-dependent E3 ubiquitin ligase that couples protein folding/refolding to protein degradation, as an interacting protein of PRMT5 via mass spectrometry. Their interaction was further verified by co-immuoprecipitation, GST pull-down, and bimolecular fluorescence complementation (BiFC) assay. In addition, we provided evidence that the CHIP/chaperone system is essential for the negative regulation of PRMT5 expression via K48-linked ubiquitin-dependent proteasomal degradation. Given that down-regulation of CHIP and overexpression of PRMT5 have been observed in several human cancers, our finding suggests that down-regulation of CHIP may be one of the mechanisms underlying PRMT5 overexpression in these cancers.

1. Introduction

Protein arginine methyltransferase 5 (PRMT5) is a type II methyltransferase that can symmetrically methylate arginine residues of histones and non-histone substrates [1]. The symmetric methylation on histone H4 at arginine 3 (H4R3) and/or histone H3 at arginine 8 (H3R8) is generally thought to result in transcriptional repression of target genes such as suppressor of tumorigenicity 7 [1,2], nonmetastatic 23 [1], p53 [3], and RBs (RB1, RBL1, RBL2) [4]; whereas methylation of non-histone substrates including E2F1, p53, RelA/p65, epidermal growth factor receptor (EGFR), RAD9, and programmed cell death 4 generates more diverse cellular effects [5,6]. For example, the methylation of E2F1 at R111 and R113 by PRMT5 reduces its ability to suppress cell growth and to promote apoptosis, conferring a survival advantage to tumor cells [7]. Also, methylation of p65 at R30 activates NF-κB signaling pathway and facilitates the expression of its target genes including tumor necrosis factor (TNF), TNF receptor-associated factor 1, interleukin-8, and interleukin 1A [8]. It has been proposed that PRMT5 functions as an oncoprotein by either silencing the expression of tumor suppressors or activating the signaling molecules that are crucial for cancer cells [5]. In fact, recent studies have shown that up-regulation of PRMT5 expression correlates with the development and progression of several human cancers, such as breast cancer [9], gastric cancer [10], colorectal cancer [7], ovarian cancer [11], leukemia, and lymphoma [2]. However, how PRMT5 expression is regulated in cancer cells remains largely unknown.

We have previously demonstrated that in human prostate cancer cells, PRMT5 can be transcriptionally activated by nuclear factor Y (NF-Y), and that the protein kinase C (PKC)/c-Fos signaling pathway negatively regulates PRMT5 expression through transcriptional down-regulation of NF-Y [12]. Recent research has also found that MYC directly up-regulates the transcription of the core small nuclear ribonucleoprotein particle (snRNP) assembly genes, in which PRMT5 is the key

Abbreviations: PRMT5, protein arginine methyltransferase 5; EGFR, epidermal growth factor receptor; TNF, tumor necrosis factor; NF-Y, nuclear factor Y; PKC, protein kinase C; Hsp90, heat shock protein 90; UPS, ubiquitin–proteasome system; CHIP, carboxyl terminus of heat shock cognate 70-interacting protein; TPR, tetratricopeptide repeat; Hsp70, heat shock protein 70; HEK293T, human embryonic kidney 293 T; CHX, cycloheximide; PMA, phorbol-12-myristate-13-acetate; GA, geldanamycin; 17-AAG, 17-(Allylamino)-17-demethoxygeldanamycin; WT, wild-type; MS, mass spectrometry; GST, glutathione S-transferase; MEP50, methylosome protein 50; WCL, whole cell lysate.

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enzymatic component [13]. In addition to the transcriptional regulation of PRMT5 expression, PRMT5 is also regulated by miR-92b/96 in mantle cell lymphoma [2]. Research from the same group also demonstrates that down-regulation of another three miRNAs (miR-19a, miR-25, and miR-32) in several lymphoid cancer cell lines leads to an increase of PRMT5 protein expression [4]. Recently, it has been observed that treatment of three different human cancer cell lines (ovarian, colon, and melanoma) with the heat shock protein 90 (Hsp90) inhibitor 17-AAG reproducibly down-regulates the expression of PRMT5 at the protein level [14]. Given the role of Hsp90 in the regulation of protein folding and degradation, it is reasonable to postulate that PRMT5 may be a putative client protein for Hsp90 [14].

Ubiquitination is one of the most important post-translational modifications that regulate diverse cellular signaling [15]. To execute the ubiquitination process, the consecutive action of three enzymes, including the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligases, is required for the attachment of ubiquitin to a substrate [16,17]. The ubiquitin–proteasome system (UPS) is often utilized to fine-tune the expression of target proteins that are associated with cancer development and progression. As a mechanism of quality control for protein folding, ubiquitin-dependent proteasomal degradation is often coupled with the molecular chaperone system to remove misfolded proteins [16,18,19]. In this system, E3 ubiquitin ligases appear to be the key regulators that function together with the chaperone system to regulate protein degradation. Carboxyl terminus of heat shock cognate 70-interacting protein (CHIP), also known as STUB1/STIP1 homology and U-Box containing protein 1, is a chaperone-dependent E3 ubiquitin ligase [20,21]. CHIP contains three tandem tetratricopeptide repeat (TPR) motifs, through which it interacts with the chaperones including heat shock protein 70 (Hsp70) and Hsp90, and a U-box domain, which is responsible for ubiquitination of the chaperone-bound substrates. Recently, CHIP has been proposed as a tumor suppressor since lower expression of CHIP promotes cell proliferation and/or inhibits apoptosis in breast cancer [22,23], gastric cancer [24], pancreatic cancer [25], and colorectal cancer [26]. Specifically, the role of CHIP in these cancers is to control the expression of several crucial proteins, such as ErbB2 [22], hypoxia-inducible factor-1a [27], c-Myc [28], p65 [26], and EGFR [25].

In the present study, we demonstrated that PRMT5 can undergo polyubiquitination both *in vivo* and *in vitro*. We also provided evidence that the E3 ubiquitin ligase CHIP couples to the molecular chaperone system (Hsp70/Hsp90) and mediates ubiquitin-dependent proteasomal degradation of PRMT5. Our work provides a new mechanism underlying PRMT5 overexpression in cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

Prostate cancer cell line LNCaP, human embryonic kidney 293 T (HEK293T), and COS-1 cells were purchased from American Type Culture Collection (ATCC) and were maintained in RPMI 1640 or DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with penicillin/streptomycin, sodium pyruvate, and L-glutamine. All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. Cycloheximide (CHX) and MG132 were purchased from Sigma. GA and 17-AAG were purchased from Tocris Bioscience.

2.2. Plasmid construction

The pCMV-Myc-PRMT5 expression plasmid was previously constructed [12] and was used as a template to generate methyltransferase activity-deficient mutant pCMV-Myc-PRMT5-R368A [29], and a series of truncated fragments covering the residues 229–637, 284–637, 352–637, and 451–637. For mutagenesis, nucleotide substitutions (from lysine/K to arginine/R) were introduced into PRMT5 using ligation PCR as described previously [12,30]. pCMV-FLAG-PRMT5 was generated by subcloning PRMT5 into pCMV-FLAG expression vector (Sigma). Various truncated mutants and single-point mutations of PRMT5 were generated using PCR or ligation PCR, and then subcloned into pCMV-FLAG or pCMV-HA (Clontech). The chaperone-interaction-deficient K30A mutant (Lysine/K to alanine/A at position 30) and E3 ubiquitin ligase activity-deficient H260Q mutant (histidine/H to glutamine/Q at position 260) for CHIP were generated using the same methods. Two truncated fragments of CHIP were amplified by PCR using primers specific for ΔU-box (forward primer: 5′-ccggaattcggatgaagggcaaggagg-3′ and reverse primer: 5′-cggggtaccgaggtaggagtgcagctc-3′) and ΔTPR (forward primer: 5′-ccggaattcggatcgcgaagaagaagcg-3′ and reverse primer: 5′-cggggtaccgtagtcctccacccagcc-3′), and then were subcloned into pCMV-FLAG. To express CHIP as a fusion with GST, the cDNA encoding CHIP was subcloned into pGEX-4 T2 vector. For BiFC plasmid construction, pCMV-Myc and pCMV-HA were used to generate pBiFC-VN155(I152L)-N and pBiFC-VC155-N vectors, followed by the subcloning of the cDNAs encoding PRMT5 and CHIP into either of these two BiFC cloning vectors. cDNAs encoding wild-type (WT) ubiquitin, ubiquitin-K48R, and ubiquitin-K63R were kind gifts from Dr. Chittaranjan Das lab (Purdue University) and were then subcloned into pCMV-HA vector. All plasmid constructs were verified by enzymatic digestion or DNA sequencing.

2.3. In vivo ubiquitination assay

Cells were co-transfected with the plasmid encoding HA-Ubiquitin and Myc-PRMT5 or its various mutants, along with plasmids encoding FLAG-CHIP or CHIP mutants for the indicated time, followed by the treatment with MG132 (10 μM) for another 6 h. Whole cell lysate (WCL) was prepared, and 500 μg of the WCL was used for immunoprecipitation (IP) using the antibodies against PRMT5, HA, and Myc, followed by the detection of respective proteins by immunoblotting (IB). For the detection of protein ubiquitination, a final concentration of 10 mM NEM (Sigma, E3876-5G) was added to the IP buffer in order to inhibit protein deubiquitination.

2.4. Co-immunoprecipitation and immunoblotting

Cells were harvested and washed twice with cold phosphate buffered saline (PBS) and then lysed by sonication in lysis buffer (10 mM Tris–HCl pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl) containing 1 mM phenylmethlysulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), protease cocktail, 25 mM okadaic acid, and 1% Triton X-100 as described previously [31]. For the preparation of soluble and insoluble samples, supernatant was collected and saved as soluble fraction, and pellets were resuspended in the same volume of lysis buffer and sonicated on ice, and the boiled pellets were saved as insoluble fraction. For co-immunoprecipitation (Co-IP), cells were treated with or without 17-AAG for 24 h, and the cell lysate was prepared by sonication in IP buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1.5 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM beta-glycerolphosphate, 1 mM PMSF, and protease cocktail), and IP was performed following the same procedure as described previously [12,32]. The antibodies used for IB analysis were anti-β-actin (Cell Signaling Technology, 8H10D10), anti-PRMT5 (Millipore, 07–405), anti-CHIP (Santa Cruz, G-2 sc-133,066), anti-FLAG M2 (Cell Signaling Technology, 9A3), anti-HA (Cell Signaling Technology, 6E2), anti-GST (BD Biosciences), and anti-Myc (GenScript, A00704-100). Secondary HRP-conjugated antibodies were purchased from Amersham Biosciences.

2.5. Mass spectrometry analysis of PRMT5 interacting proteins in LNCaP cells

For the identification of PRMT5 interacting proteins using mass spectrometry, LNCaP cells were transfected with the plasmids encoding FLAG-PRMT5 and HA-Ubiquitin for 42 h, followed by the treatment with MG132 for another 6 h. WCL was used for IP of FLAG-PRMT5 with anti-FLAG antibody, or the control IgG, followed by trypsin digestion and quantitative mass spectrometry analysis as described before [33]. Three independent experiments were performed, and E3 ligases that were specifically identified in the anti-FLAG immunoprecipitates but not in the IgG control were considered as putative E3 ligases for PRMT5 interaction.

2.6. GST pull-down assay

pGEX-4 T2-CHIP was transformed into *Escherichia coli* strain BL21, and a single colony of the transformed bacteria was inoculated into 200 ml LB medium and cultured at 37 °C until the optical density value reached 0.6. CHIP expression was induced by adding 1.0 mM isopropyl-beta-D-thiogalactopyranoside into the culture for 4 h. For cell lysate preparation, pelleted bacteria were resuspended in ice cold lysis buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl) and disrupted by sonication, followed by centrifugation at 15,000 × *g* for 30 min at 4 °C. For GST pull-down assay, plasmid encoding Myc-PRMT5 was transfected into HEK293T cells using FuGENE 6 following the manufacturer's instructions and incubated for 24 h. The transfected cells were then lysed, and WCL was prepared. Approximately 500 μg of WCL was incubated with the same molar ratio of GST and GST-CHIP at 4 °C for overnight, followed by the incubation with glutathione-Sepharose beads (GE Healthcare) for another 2 h. The beads were washed three times with lysis buffer and boiled in 2 × SDS loading buffer and subjected to SDS-PAGE gel analysis [34].

2.7. BiFC assay

BiFC assay was performed essentially the same as previously described to analyze the interaction between PRMT5 and CHIP in COS-1 cells [35]. Briefly, COS-1 cells were grown on coverslips in a 12-well plate for 24 h, and the BiFC plasmids encoding Myc-VN155-PRMT5 and HA-VC155-CHIP, along with FLAG-Cerulean, were co-transfected into COS-1 cells for 24 h. Cells were then fixed with 3.7% paraformaldehyde and stained with 4′6-Diamidino-2-Phenylindole (DAPI) for 5 min at room temperature (RT) under dark condition. The fluorescent images were acquired by Nikon A1 confocal microscope.

2.8. Luciferase assay

HEK293T cells were transiently transfected with 1 μg of pCMV-FLAG (Vector) or pCMV-FLAG-CHIP (CHIP), along with 500 ng of the PRMT5 proximal promoter reporter gene, plus 100 ng of pRL-TK for 24 h using Lipofectamine® 3000 Transfection Reagent (Invitrogen), and the relative luciferase activity was determined using Dual-Luciferase® Reporter Assay system (Promega) as described previously [12].

2.9. Reverse transcription and real-time PCR

For real-time PCR analysis, total RNA was purified using TRIzol® Plus RNA Purification Kit (Life Technologies), and 2 μg of RNA was then reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Invitrogen) according to the manufacturer's protocol. Human PRMT5 and GAPDH primers used for real-time PCR were the same as described previously [12]. For real-time PCR, StepOne Real-Time PCR (Applied Biosystems) was performed by using SYBR Select Master Mix. All real-time PCR reactions were performed in triplicate with at least three independent experiments, and the relative expression of each gene was normalized to GAPDH [36].

2.10. RNA interference

Endogenous CHIP was depleted in cells using siGENOME Human STUB1/CHIP (10273) siRNA SMARTpool (Dharmacon, Lafayette, CO), and siGENOME Non-Targeting siRNA Pool (Dharmacon, Lafayette, CO) was used as a negative control. For siRNA experiments, the indicated siRNA was transfected into HEK293T cells using DharmaFECT 1 Transfection Reagent (Dharmacon) according to the manufacturer's protocol. After cells were transfected for 72 h, WCL was prepared, and the ubiquitination pattern or the expression level of CHIP was analyzed by immunoblotting.

2.11. Analysis of cell apoptosis by flow cytometry

Plasmid encoding FLAG-CHIP (or Vector only) was transfected into cells for 48 h, followed by the treatment of 17-AAG for another 24 h. Both floating and adherent cells were collected for flow cytometry analysis using Annexin V-APC/7-amino-actinomycin D Apoptosis Detection Kit (KeyGEN Biotechnology, Nanjing, China). Briefly, HEK293T cells were trypsinized and washed with filtered PBS twice, resuspended in 200 μl binding buffer with 2 μl Annexin V-APC, and then incubated at RT for 15 min. Supernatant was gently removed after 300 × g centrifugation for 2 min, followed by adding 2 μl of 7-ADD into 200 μl binding buffer and incubated at RT for 5 min in the dark. At least 50,000 cells were resuspended in 800 μl of PBS. Three independent experiments were performed using a BD Accuri C6 flow cytometer at a low flow rate with a minimum of 1×10^4 cells, and the percentage of apoptotic cells was determined.

2.12. Cell growth analysis

To determine the role of CHIP in 17-AAG-induced cell growth inhibition, HEK293T cells were seeded and grown on coverslips in a 6-well plate at a cell density of 1×10^5 cells/well, and siRNA control (siCon) or siCHIP was transfected into cells for 48 h using DharmaFECT 1 Transfection Reagent, followed by the treatment with or without 17AAG (100 nM) for another 24 h. Total cell number was counted using hemocytometer, and the percentage of cell growth over the control was determined [12].

2.13. Sequence alignment and visualization of PRMT5 structure

Sequence alignment and ubiquitination site prediction were performed using several online alignment and prediction software (http://bdmpub.biocuckoo.org/prediction.php, http://www.ubpred.org/, and http://protein.cau.edu.cn/cksaap_ubsite/), and the crystal structures of PRMT5 and MEP50 were retrieved from PDB database (accession code 4GQB) and processed by PyMOL software (http://www.pymol.org/). The illustration of protein domain of PRMT5 and CHIP was created using DOG1.0 software [37].

2.14. Statistical analysis

The GraphPad Prism 6 Software (Graphpad Software, San Diego, CA, USA) was used to perform all statistical analysis. Data were presented as mean ± SD from at least three independent experiments. Comparison between two groups was conducted by using Student's t test. Two-way ANOVA was used to compare the means of two independent variables, followed by Tukey's post-hoc test. p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. PRMT5 undergoes polyubiquitination in LNCaP cells

We have previously shown that PRMT5 is transcriptionally activated by NF-Y in LNCaP prostate cancer cells, and that treatment of cells with the PKC activator phorbol-12-myristate-13-acetate (PMA) down-regulates PRMT5 expression [12]. During the course of these experiments, we noticed that PMA-induced PRMT5 down-regulation appeared to be partially reversed by the proteasome inhibitor MG132 (Fig. 1A), suggesting that PRMT5 might undergo proteasomal degradation. Given that polyubiquitination is a prerequisite for the proteasomal degradation of many cytosolic proteins [38], we sought to determine whether PRMT5 is subjected to polyubiquitination. To this end, LNCaP cells transfected with the plasmid encoding HA-Ubiquitin were treated with or without MG132, followed by immunoprecipitation of endogenous PRMT5 with anti-PRMT5 antibody. Indeed, polyubiquitination of endogenous PRMT5 was readily detectable in the absence of MG132, and the presence of MG132 further enhanced the polyubiquitination of PRMT5 (Fig.1B). This result provides evidence that endogenous PRMT5 is polyubiquinated. To determine whether exogenously expressed PRMT5 also undergoes polyubiquitination, we co-expressed Myc-PRMT5 with HA-Ubiquitin (or HA-Vector) in LNCaP cells in the presence of MG132, and then immunoprecipitated Myc-PRMT5 with anti-Myc antibody followed by immunoblotting of HA-Ubiquitin with anti-HA antibody. As shown in Fig. 1C, Myc-PRMT5 was clearly polyubiquitinated. A reverse immunoprecipitation using anti-HA antibody was performed to further confirm the polyubiquitination of the exogenously expressed Myc-PRMT5 in LNCaP cells (Fig. 1D). Taken together, we conclude that PRMT5 can undergo polyubiquitination at both endogenous and exogenous level in LNCaP cells.

3.2. PRMT5 polyubiquitination involves multiple lysine residues

Since covalent attachment of multiple ubiquitin molecules to specific lysine residues of target proteins is a prerequisite for recognition and subsequent degradation by proteasome [39], we were interested in identifying the lysine residues that are responsible for PRMT5 ubiquitination. We generated a series of deletion mutants based on PRMT5 structure (Fig. 2A, top) and co-expressed them with HA-Ubiquitin in LNCaP cells to map the ubiquitination sites. As shown in Fig. 2A, all of these mutants appeared to undergo polyubiquitination in the presence or absence of MG132 treatment. Significantly, the PRMT5 mutants 229–637, 284–637, and 352–637 were highly ubiquitinated when compared with full-length PRMT5, whereas the ubiquitination pattern of the PRMT5 mutant 451–637 remained unchanged in the presence of MG132 treatment, suggesting that the major ubiquitination sites of PRMT5 are located between residues 229 and 451. Based on the crystal structure of PRMT5, we then focused on

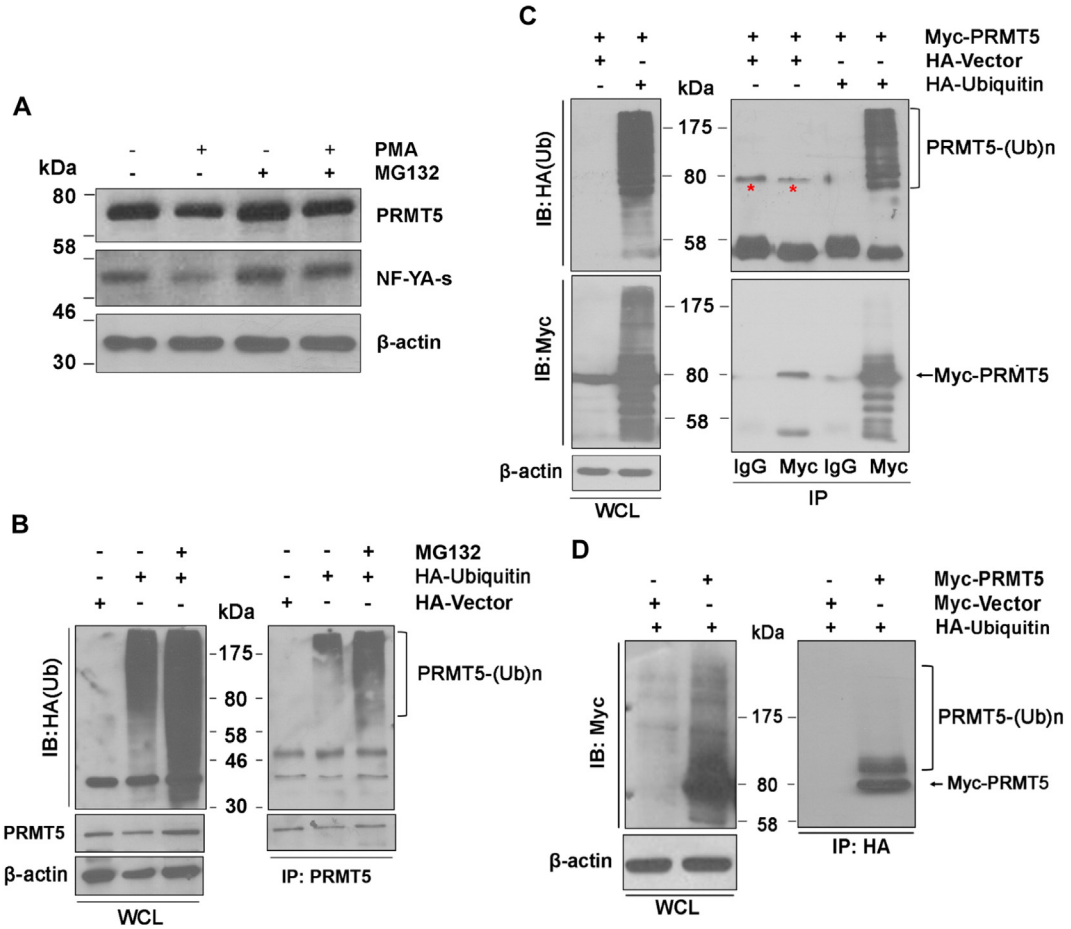

Fig. 1. Ubiquitination of PRMT5 in LNCaP cells. (A) Proteasome inhibitor MG132 partially restores PMA-induced reduction of PRMT5 expression. LNCaP cells were treated with the PKC activator PMA (100 nM) in the presence or absence of MG132 (10 μM) for 24 h, and the whole cell lysate (WCL) was prepared and subjected to immunoblotting (IB). NF-YA-s (a positive control) represents the short isoform of NF-YA. (B) Ubiquitination of endogenous PRMT5 in LNCaP cells. LNCaP cells were transfected with HA-Vector or the plasmid encoding HA-Ubiquitin for 42 h, followed by the treatment with DMSO (−) or MG132 (+) for another 6 h. WCL was immunoprecipitated with anti-PRMT5 antibody and probed with anti-HA or anti-PRMT5 antibody. (C) Ubiquitination of exogenous PRMT5 in LNCaP cells. Myc-PRMT5 was co-expressed with either HA-Vector or HA-Ubiquitin in LNCaP cells for 48 h, and WCL was immunoprecipitated with IgG or anti-Myc antibody, respectively, followed by immunoblotting of β-actin, HA-Ubiquitin, and Myc-PRMT5. * indicates non-specific band. (D) HA-Ubiquitin was co-expressed with either Myc-Vector or Myc-PRMT5 in LNCaP cells for 48 h, and WCL was immunoprecipitated with anti-HA antibody, followed by immunoblotting with β-actin and Myc antibodies. PRMT5-(Ub)n in B, C, and D indicates polyubiquitination of PRMT5.

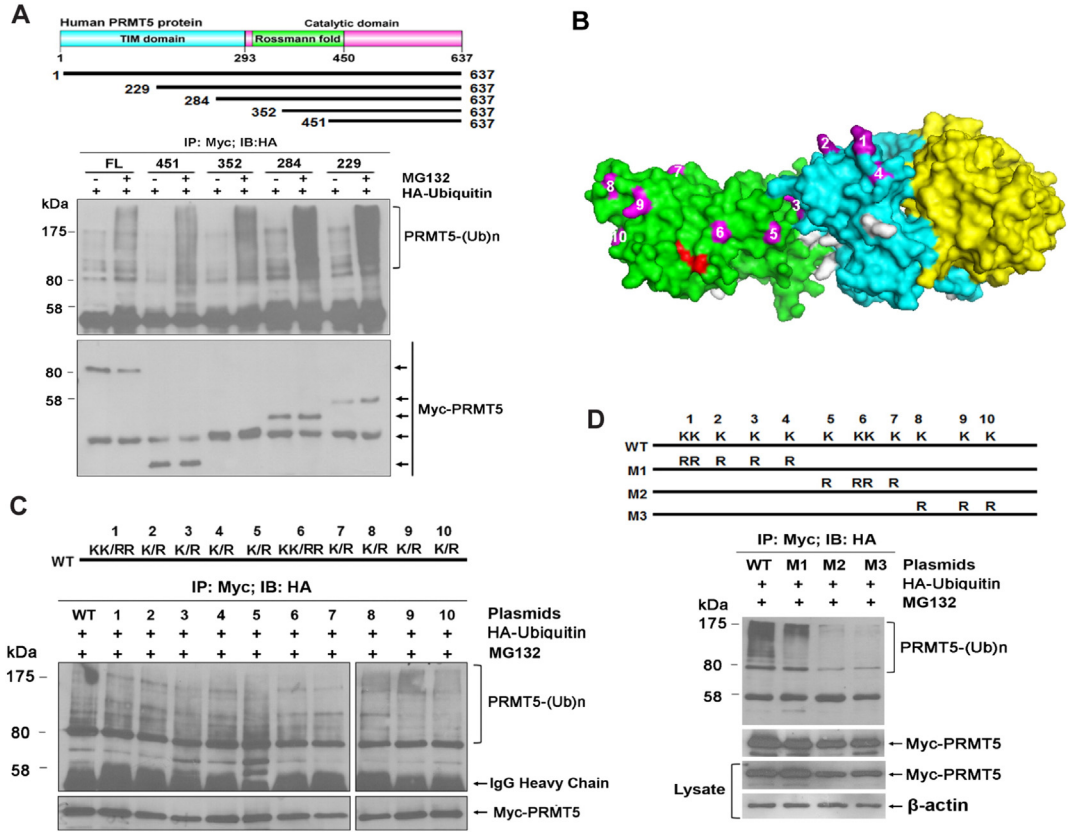

Fig. 2. Multiple lysine residues are involved in the polyubiquitination of PRMT5. (A) A series of PRMT5 truncated mutants were generated (top) and were co-expressed with HA-Ubiquitin in the presence or absence of MG132 in LNCaP cells, and the whole cell lysate (WCL) was then immunoprecipitated with anti-Myc antibody and subjected to immunoblotting for HA-Ubiquitin detection using anti-HA. The membrane was then stripped and re-probed with antibody against Myc. (B) Illustration of predicted surface-exposed lysine residues in PRMT5. Lysine residues exposed on the surface of PRMT5 are highlighted in white with an indicated number. Green represents the catalytic domain of PRMT5 while the light blue indicates the TIM domain of PRMT5, the yellow represents MEP50. (C) The effect of K/R mutations on PRMT5 polyubiquitination. The individual lysine (K) was mutated to arginine (R), and the mutants were co-expressed with HA-Ubiquitin in LNCaP cells in the presence of MG132 for 48 h, and the WCL was subjected to IP with anti-Myc antibody and IB for the detection of PRMT5 polyubiquitination with anti-HA antibody. (D) The polyubiquitination of the indicated PRMT5 mutants was similarly analyzed as described in C.

ten most surface-exposed lysine (K) residues (highlighted in Fig. 2B) within the region of residues 229–451. We mutated these lysine residues to arginine (R) at the indicated sites, including positions at 240 and 241 (1), 248 (2), 259 (3), 275 (4), 302 (5), 329 and 333 (6), 343 (7), 354 (8), 380 (9), and 387 (10). The expression level of all mutants was comparable; however, mutated individual lysine did not significantly change the ubiquitination pattern of PRMT5 (Fig. 2C). Next, we mutated the first five K (M1), the middle four K (M2), and the last three K (M3) to R in combination. As shown in Fig. 2D, all three mutants (M1, M2, and M3) showed a dramatic decrease of polyubiquitination, suggesting that multiple lysine residues might be involved in the polyubiquitination of PRMT5.

3.3. Co-chaperone E3 ubiquitin ligase CHIP interacts with PRMT5

E3 ubiquitin ligases are critical regulators of the ubiquitination process for specific substrates [40]. To identify E3 ubiquitin ligases specific for PRMT5, HA-Ubiquitin was co-expressed with FLAG-PRMT5 in LNCaP cells for 42 h and treated with MG132 for another 6 h, followed by immunoprecipitation using anti-FLAG antibody or IgG. The immunoprecipitates were subjected to mass spectrometry analysis. Two ubiquitin E3 ligases, CHIP and TRIM21, and one sumo E3 ligase, RanBP2, were specifically identified from three independent experiments (Table 1). Given the high coverage of CHIP, we selected CHIP for further validation as a potential interacting protein of PRMT5. Since HEK293T cells have a higher transfection efficiency, we co-expressed Myc-PRMT5 with FLAG-CHIP in HEK293T cells for 48 h in the presence of MG132 and then performed immunoprecipitation with anti-FLAG antibody. Compared with FLAG-Vector or IgG control, Myc-PRMT5 was co-immunoprecipitated with FLAG-CHIP (Fig. 3A), suggesting the specific interaction between Myc-PRMT5 and FLAG-CHIP in cells. Their interaction was further validated using GST pull-down assays (Fig. 3B), as evidenced by the enrichment of PRMT5 by GST-CHIP when compared with GST only. To ascertain the physiological interaction between CHIP and PRMT5, the WCL from HEK293T was prepared and subjected to reciprocal co-immunoprecipitation with either anti-CHIP antibody or anti-PRMT5 antibody. As shown in Fig. 3C, PRMT5 and CHIP were specifically co-immunoprecipitated with either antibody, demonstrating that PRMT5 and CHIP also interact with each other at the endogenous level. Since CHIP and PRMT5 can be both localized to the

Table 1
Identification of putative E3 ligases for PRMT5 by mass spectrometry.

Accession	Gene	Description	Coverage	Unique Peptides	Repeatability
H3BUD0	**CHIP**	**E3 ubiquitin ligase**	**18.92**	**3**	**3**
F5H012	**TRIM21**	**E3 ubiquitin ligase**	**10.53**	**4**	**3**
P49792	**RanBP2**	**E3 SUMO ligase**	**2.73**	**4**	**2**

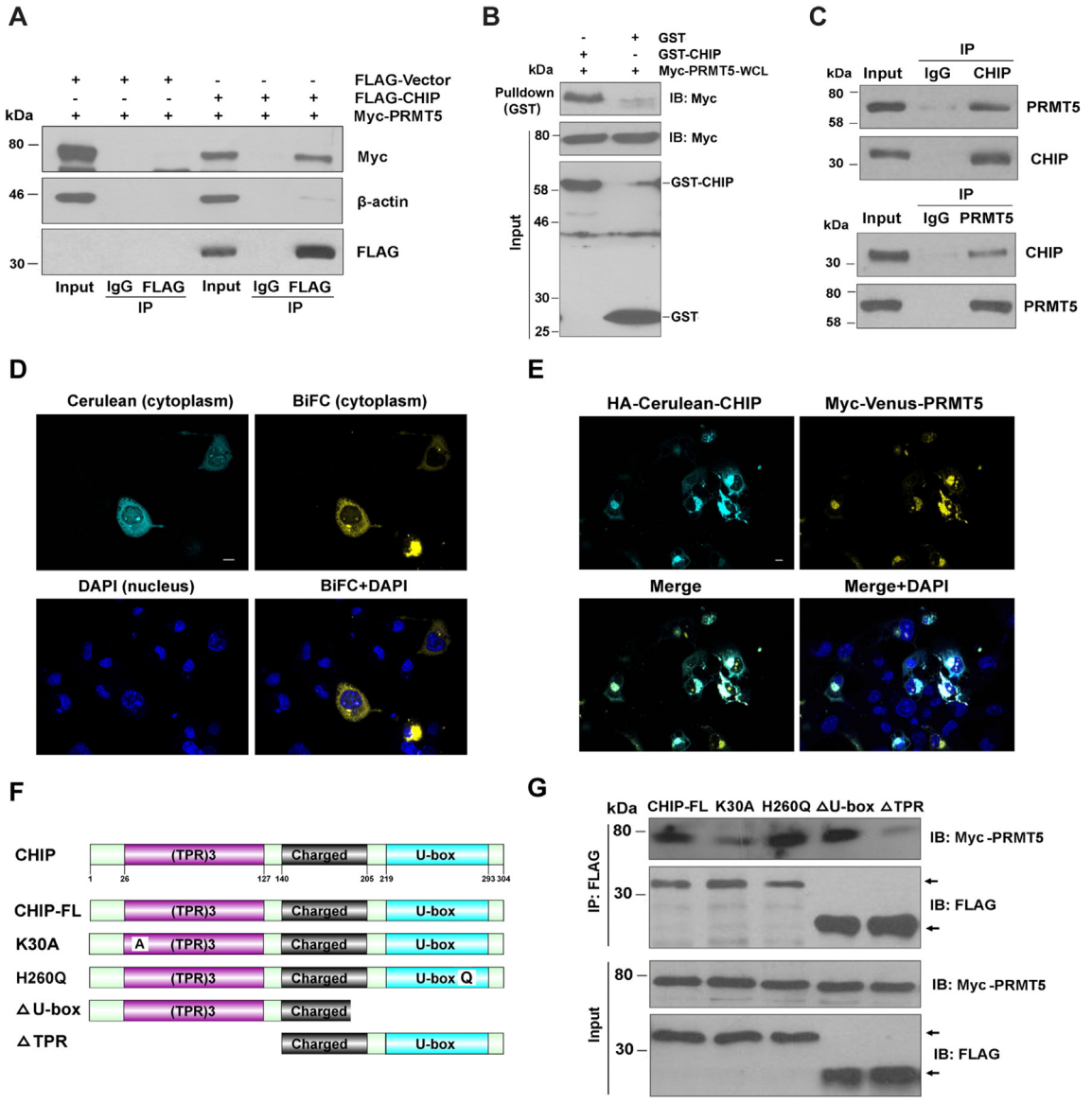

Fig. 3. CHIP interacts with PRMT5 both *in vitro* and *in vivo*. (A) FLAG-CHIP interacts with Myc-PRMT5 in HEK293T cells. Myc-PRMT5 was co-expressed with FLAG-vector or FLAG-CHIP in HEK293T cells for 48 h, and MG132 was applied for another 6 h. Whole cell lysate (WCL) was prepared and immunoprecipitated with IgG or anti-FLAG antibody for overnight, and immunoprecipitates were analyzed by immunoblotting using Myc, β-actin, and FLAG antibodies. Input: 5% of WCL. (B) GST-CHIP interacts with Myc-PRMT5 *in vitro*. GST and GST-CHIP were expressed in *E. coli* and then immobilized to glutathione agarose beads. The same amount of HEK293T WCL containing overexpressed Myc-PRMT5 (Myc-PRMT5-WCL) was then incubated with GST or GST-CHIP, and the pull-down fraction was used for immunoblotting analysis. (C) HEK293T cells were treated with 17-AAG (100 nM) for 24 h, along with MG132 treatment for another 6 h. WCL was used for IP using antibodies against CHIP or PRMT5, and IgG was used as a negative control. (D) The two BiFC plasmids encoding the Myc-VN155-PRMT5 and HA-VC155-CHIP along with pFLAG-Cerulean were co-transfected into COS-1 cells for 24 h. Shown are representative fluorescent images of transfected cells (Cerulean) and the interaction between PRMT5 and CHIP (BiFC). Nuclei were stained with DAPI. Scale bar is 5 μm. (E) Co-localization of PRMT5 and CHIP in cells. Myc-Venus-PRMT5 was co-expressed with HA-Cerulean-CHIP in COS-1 cells for 24 h, and their co-localization was analyzed by confocal microscopy. Scale bar is 5 μm. (F) A schematic for CHIP and its mutants. CHIP-FL represents full-length CHIP, which includes three major domains: $(TPR)_3$, Charged, and U-box domains. K30A indicates chaperone-interaction-deficient mutant; H260Q indicates ubiquitination-deficient mutant; ΔU-box represents U-box deletion mutant; ΔTPR represents TPR deletion mutant. (G) TPR domain of CHIP is required for PRMT5 interaction. Myc-PRMT5 was co-expressed with FLAG-CHIP or its mutants in HEK293T cells for 48 h and treated with MG132 for another 6 h. WCL was used for immunoprecipitation as described above. Arrow indicates FLAG-CHIP or its mutants.

cytoplasm and nucleus [41], we then determined where they interact in cells using bimolecular fluorescence complementation (BiFC) technique [35,42]. Given that COS-1 cells have a better cytoplasm/nucleus ratio for visualization of subcellular localizations, we transiently transfected plasmids encoding HA-VC155-CHIP and Myc-VN155-PRMT5 along with a plasmid encoding FLAG-Cerulean into COS-1 cells for 24 h. As shown in Fig. 3D, the Venus signal (BiFC signal) in the transfected cells was predominantly localized in the cytoplasm, suggesting that the interaction between CHIP and PRMT5 likely occurred in the cytoplasm. In line with this, we also found that both CHIP and PRMT5 were co-localized in the cytoplasm by co-expressing them fused to full-length Cerulean and Venus, respectively (Fig. 3E).

CHIP contains a TPR domain involved in the interaction with chaperones at the N-terminus, a U-box domain that possesses ubiquitin ligase activity at the C-terminus, and a linker known as charged domain in between [20]. In order to determine which region of CHIP is required for PRMT5 interaction, we then generated a series of CHIP mutants (chaperone interaction-deficient mutant K30A, ubiquitination-deficient mutant H260Q, and TPR or U-box deletion mutant), to map the PRMT5 interaction domain in CHIP (Fig. 3F). We co-expressed these CHIP mutants as FLAG fusion proteins with Myc-PRMT5 in HEK293T cells and performed immunoprecipitation with anti-FLAG antibody and immunoblotting for Myc-PRMT5 with anti-Myc antibody. Although both H260Q and U-box deletion (ΔU-box) mutants co-immunoprecipitated comparable

amount of Myc-PRMT5 when compared with the CHIP-FL, the binding of Myc-PRMT5 to the K30A and TPR deletion (ΔTPR) mutants was almost abolished (Fig. 3G). This result suggests that the TPR domain of CHIP is necessary for the interaction with PRMT5 and that the binding of PRMT5 and chaperons to CHIP may share the same binding motif. However, the interaction between CHIP and PRMT5 is independent of the E3 ligase activity of CHIP. Taken together, these results demonstrate that CHIP and PRMT5 can interact both *in vitro* and *in vivo*, and the interaction likely occurs in the cytoplasm.

3.4. CHIP negatively regulates PRMT5 expression

The finding that CHIP interacts with PRMT5 prompted us to determine whether CHIP regulates PRMT5 expression. We first co-expressed Myc-PRMT5 with increasing amounts of FLAG-CHIP in HEK293T cells for 48 h and then detected the expression of Myc-PRMT5. As shown in Fig. 4A, FLAG-CHIP dose-dependently decreased Myc-PRMT5 protein expression. We also confirmed that there was no significant effect of FLAG-CHIP on the PRMT5 promoter-driven reporter gene activity and PRMT5 mRNA

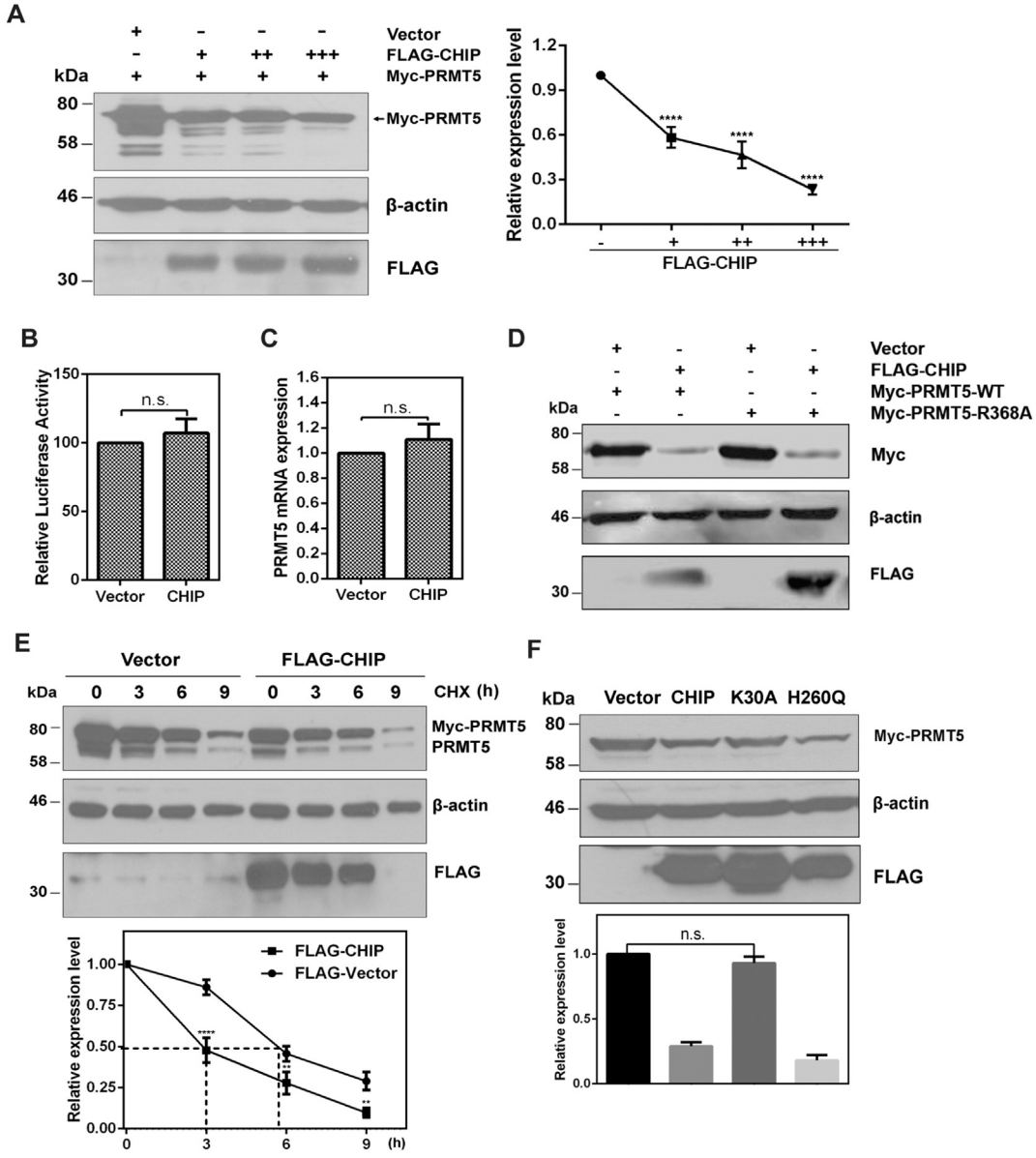

Fig. 4. CHIP negatively regulates PRMT5 expression. (A) Overexpression of CHIP dose-dependently decreases PRMT5 expression. pMyc-PRMT5 was co-transfected with pFLAG-Vector (Vector) or an increasing amount of pFLAG-CHIP into HEK293T cells for 48 h. Antibodies against PRMT5, FLAG, and β-actin were used for immunoblotting. Representative blots from three independent experiments are shown, and the images were analyzed by Image J software and relative expression of Myc-PRMT5 is presented as mean ± SD (on the right), $^{****}p < 0.0001$ one-way ANOVA. (B) Overexpression of CHIP has no effect on the PRMT5 promoter activity. One microgram of pFLAG-Vector (Vector) or pFLAG-CHIP (CHIP) was co-transfected with 0.5 μg of the PRMT5 proximal promoter reporter gene, along with 100 ng of pRL-TK into HEK293T cells for 24 h, and the relative luciferase activity was determined and analyzed. (C) Overexpression of CHIP has no effect on PRMT5 mRNA expression. Three micrograms of pFLAG-Vector (Vector) or pFLAG-CHIP (CHIP) was transfected into HEK293T cells for 24 h, and the PRMT5 mRNA level was determined by real-time PCR. (D) CHIP promotes degradation of PRMT5 and its methyltransferase activity-deficient mutant. pMyc-PRMT5-WT or pMyc-PRMT5-R368A was co-transfected with pFLAG-CHIP into HEK293T cells for 48 h. Antibodies against Myc, FLAG, and β-actin were used for immunoblotting (IB). (E) CHIP promotes the turnover rate of PRMT5 in HEK293T cells. HEK293T cells were transfected with either pFLAG-Vector or pFLAG-CHIP along with Myc-PRMT5 for 36 h, followed by the treatment with 10 μg/mL cycloheximide (CHX) for different times, and the turnover rate of Myc-PRMT5 was determined by immunoblotting. Representative results are shown (Top). Bottom: Quantitative result analyzed by Image J is presented as means ± SD from three independent experiments. Dashed line indicates the time required for exogenous PRMT5 being degraded to 50%. Statistical significance ($^{**}p < 0.01$; $^{****}p < 0.0001$) was determined by two-way ANOVA followed by Tukey's test. (F) The effect of CHIP and its mutants on the expression of PRMT5. Myc-PRMT5 was co-expressed with CHIP or CHIP mutants (K30A and H260Q) for 48 h, and the expression level of Myc-PRMT5 was determined by immunoblotting. n.s. in B, C, and F indicates no significance (Student's t test).

expression (Fig. 4B, C). Similarly, the expression of the methyltransferase activity-deficient mutant Myc-PRMT5-R368A was also down-regulated by FLAG-CHIP, suggesting that CHIP-mediated degradation of PRMT5 is independent of the catalytic activity of PRMT5 (Fig. 4D). Next, we sought to investigate the impact of CHIP expression on the half-life of PRMT5. FLAG-CHIP or FLAG-Vector was co-expressed with Myc-PRMT5 in cells for 36 h, and treatment of CHX was applied for the indicated times. As shown in Fig. 4E and Supplementary Fig. S1, CHIP expression (though vanished at 9 h) reduced the half-life of Myc-PRMT5 from 5.5 h to 3 h, suggesting that CHIP can promote PRMT5 degradation. The identification of PRMT5 as a substrate of CHIP for proteasomal degradation is particularly intriguing, given that many proteins regulated by CHIP also require the molecular chaperone system Hsp90/Hsp70 for protein folding [20,21]. We next determined whether the two CHIP mutants K30A and H260Q might affect the expression of PRMT5. Interestingly, we found that both CHIP and H260Q, but not K30A mutant, significantly attenuated Myc-PRMT5 expression (Fig. 4F), suggesting that the molecular chaperone system is required for PRMT5 recognition and its subsequent degradation by CHIP.

3.5. CHIP mediates the down-regulation of PRMT5 expression and cell growth inhibition by 17-AAG

The molecular chaperone proteins (Hsp90 and Hsp70) cooperate with the ubiquitination/proteasomal system to regulate the degradation of unfolded or misfolded proteins. CHIP is one of the major E3 ubiquitin ligases involved in this ubiquitin/molecular chaperone system [20,43,44]. Our result that the K30A mutant failed to decrease PRMT5 expression is consistent with previous reports that PRMT5 is a client protein of Hsp90 [14,45]. This led us to hypothesize that the degradation of PRMT5 may be regulated by the ubiquitin/molecular chaperone system involving CHIP, Hsp90, and Hsp70. In support of this hypothesis, we indeed found that Hsp90 inhibitors 17-AAG and GA, both of which target Hsp90 ATPase binding domain, dose-dependently decreased PRMT5 protein expression in HEK293T cells (Fig. 5A) and in LNCaP cells (Fig. 5B). Further, overexpression of CHIP enhanced 17-AAG-mediated down-regulation of PRMT5 (Fig. 5C). In addition, overexpressed FLAG-CHIP increased 17-AAG-induced cell death from 14.05% to 23.39% (Fig. 5D). To understand the role of endogenous CHIP in the regulation of PRMT5 expression, siRNA SMARTpool targeting CHIP was used to knock down CHIP in HEK293T cells. Significantly, knockdown of CHIP completely inhibited 17-AAG-induced down-regulation of PRMT5 (Fig. 5E), indicating that PRMT5 expression can be regulated by the ubiquitin/molecular chaperone system in cells. We next sought to determine the effect of CHIP on 17-AAG-induced cell growth inhibition/cell death. 17-AAG indeed significantly inhibited cell growth, which is consistent with previous reports [23,46], and knockdown of CHIP partially rescued cell growth inhibition by 17-AAG (Fig. 5F). Taken together, these results suggest that 17-AAG-induced cell growth inhibition/cell death is likely mediated by CHIP-dependent down-regulation of PRMT5 expression.

3.6. CHIP promotes PRMT5 degradation through K48-linked ubiquitination

CHIP is an E3 ubiquitin ligase that mediates protein degradation by ubiquitinating its substrates [47]. Since PRMT5 undergoes ubiquitination and CHIP negatively regulates PRMT5 expression, we were interested in determining whether PRMT5 is subjected to CHIP-mediated proteasomal degradation. To this end, FLAG-CHIP and Myc-PRMT5 were co-expressed in the absence or presence of the proteasome inhibitor MG132. As shown in Fig. 6A, treatment with MG132 attenuated the inhibitory effect of FLAG-CHIP on Myc-PRMT5 expression (Fig. 6A), suggesting that the down-regulation of PRMT5 expression by CHIP is mainly through the proteasomal degradation pathway. To demonstrate that CHIP is capable of ubiquitinating PRMT5, we performed *in vivo* ubiquitination assays in HEK293T cells by transiently co-expressing Myc-PRMT5 with FLAG-CHIP in the presence of HA-Ubiquitin. Immunoprecipitation results showed that overexpression of CHIP increased the ubiquitination of PRMT5 when compared with FLAG-Vector only (Fig. 6B). However, both K30A and H260Q mutants had a reduced activity when compared with FLAG-CHIP (Fig. 6B), suggesting that the chaperone binding activity of CHIP and the U-Box region are required for CHIP-induced ubiquitination of PRMT5. However, H260Q mutant not only decreased PRMT5 expression (Fig. 4F) but also abolished the ubiquitination of PRMT5 (Fig. 6B), and this led us to investigate whether PRMT5 moves to the insoluble fraction as suggested previously [48,49]. As shown in Fig. 6C, H260Q mutant did not increase the level of insoluble PRMT5.

In contrast, knockdown of CHIP decreased 17-AAG-induced polyubiquitination of PRMT5 (Fig. 6D), indicating the necessity of CHIP in ubiquitinating PRMT5. Since CHIP can function either as a partner of Ubc13-Uev1a to induce the formation of K63-linked polyubiquitin chains [50], or a mediator for K48-linked proteasomal degradation [41], we next sought to determine which types of ubiquitination may occur in PRMT5. To this end, two ubiquitin mutants, HA-Ubiquitin-K48R (HA-Ub-K48R) and HA-Ubiquitin-K63R (HA-Ub-K63R), were co-expressed with Myc-PRMT5 in the presence of FLAG-CHIP for 48 h, and anti-Myc antibody was used for immunoprecipitation. As shown in Fig. 6E, a substantially reduced ubiquitination of PRMT5 was observed when Myc-PRMT5 was co-expressed with HA-Ub-K48R, but not HA-Ub-K63R, when compared with HA-Ub-WT, demonstrating that CHIP mediates K48-linked polyubiquitination of PRMT5. These results further support our finding that the CHIP/chaperone system (Hsp90/Hsp70) is involved in proteasomal degradation of PRMT5.

4. Discussion

PRMT5 is an emerging arginine methyltransferase that can epigenetically suppress the transcription of tumor suppressor genes and regulate the function of several signaling molecules through symmetrically dimethylating arginine residues of histones and non-histone substrates [51,52]. Recently, overexpression of PRMT5 has been demonstrated to promote cell growth or inhibit cell death in multiple cancer cell lines, and is correlated with cancer development and progression in cancer patients [4,7,9,11,53]. The de-regulation of PRMT5 expression may occur at four different levels including transcription, post-transcription, translation, and post-translation. We and others have previously demonstrated that PRMT5 can be transcriptionally activated by NF-Y [12] or post-transcriptionally regulated by miR-92b/96 [2]. However, whether the expression of PRMT5 can be regulated at post-translational level remains elusive. In the present study, we first showed that PRMT5 undergoes polyubiquitination and further demonstrated that CHIP as an E3 ubiquitin ligase interacts with PRMT5 and targets PRMT5 for ubiquitin-dependent proteasomal degradation. Results also revealed that 17-AAG-induced cell death and PRMT5 down-regulation are mediated through a CHIP-dependent mechanism.

Ubiquitination is a common type of post-translational modifications (PTMs) that regulate various cellular processes. The functional consequences of protein ubiquitination are highly dependent on the ubiquitination pattern (monoubiquitination vs polyubiquitination) and the ubiquitination linkage types [16]. At present, eight inter-ubiquitin linkage types such as K6, K11, K27, K29, K33, K48, K63, and linear ubiquitination have been reported [17,39,54]. Among them, K63 and K48 are the two most well-known ubiquitin-linked types. K63 ubiquitin linkage is involved in protein trafficking, and K48 ubiquitin linkage leads to proteasomal degradation [54]. Accumulated evidence suggests that CHIP can function as an E3 ubiquitin ligase and thereby is responsible for fine-tuning protein homeostasis through K48-linked proteasomal degradation [54,55]. Consistent with this, our results suggest that CHIP is required for ubiquitinating and targeting PRMT5 for proteasomal degradation. Several lines of evidence from our study support this conclusion. First, PRMT5 could undergo polyubiquitination, which is a prerequisite for proteasomal degradation

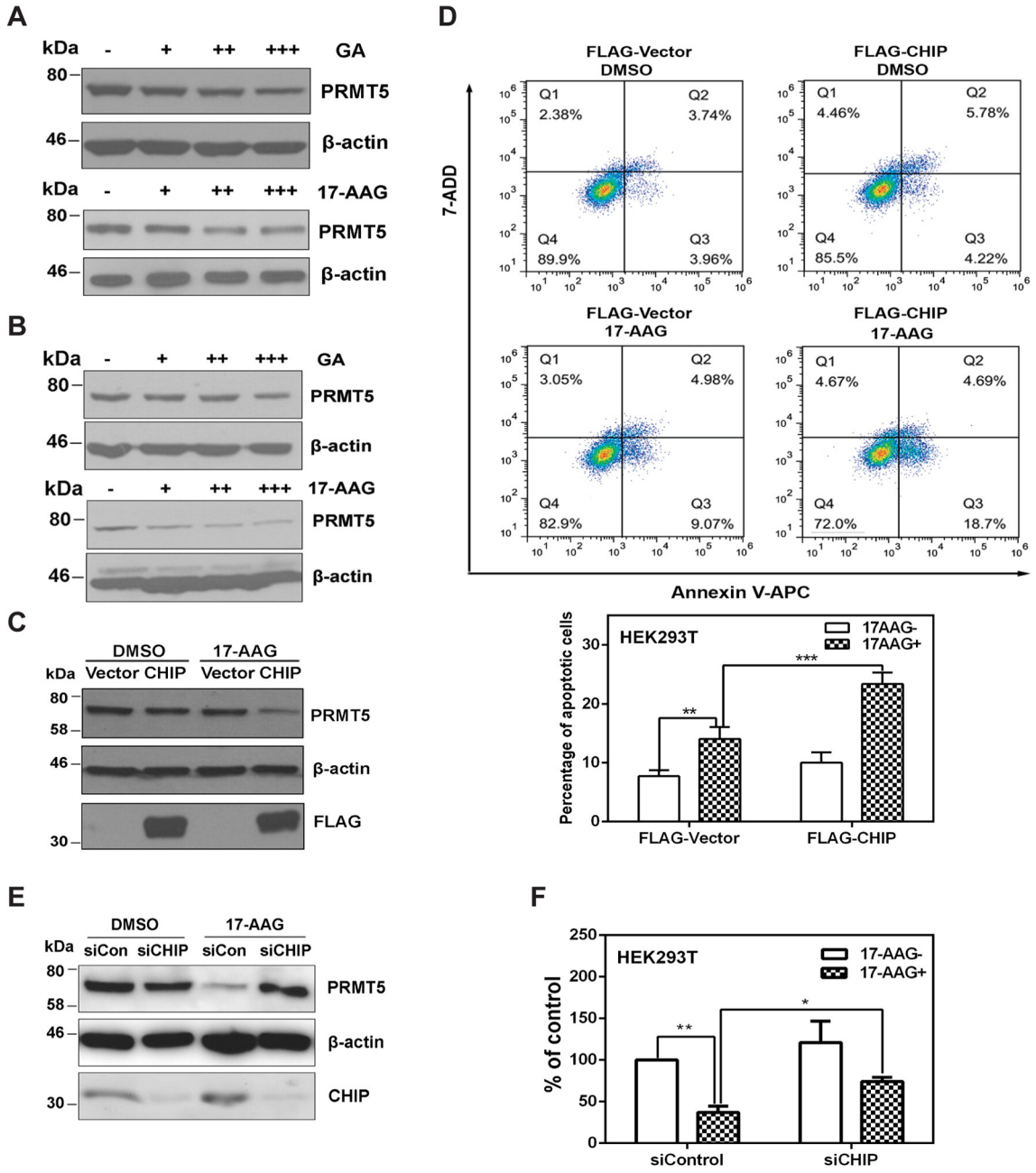

Fig. 5. CHIP mediates the down-regulation of PRMT5 expression and cell growth inhibition by 17-AAG. (A and B) Hsp90 inhibitors dose-dependently inhibit PRMT5 expression in HEK293T and LNCaP cells. HEK293T (A) and LNCaP cells (B) were treated with increasing amounts of 17-AAG (1 nM–100 nM) or GA (10 nM–1 μM) for 24 h, and the whole cell lysate (WCL) was subjected to immunoblotting (IB). (C) Overexpression of CHIP enhances the down-regulation of PRMT5 induced by 17-AAG. HEK293T cells were either transfected with pFLAG-Vector (Vector) or pFLAG-CHIP (CHIP) for 48 h, and then treated with 17-AAG for another 24 h before preparing WCL for IB. (D) Overexpression of CHIP increased 17-AAG-induced apoptosis in HEK293T cells. HEK293T cells were either transfected with pFLAG-Vector (Vector) or pFLAG-CHIP (CHIP) for 48 h, followed by the treatment with 17-AAG for another 24 h. Both floating and adherent cells were collected and labeled with Annexin V-APC and 7-amino-actinomycin D (7-ADD) for flow cytometry analysis. The percentage of apoptotic cells (Q2 + Q3) was calculated and normalized to the Vector control, and the percentage of apoptotic cells is represented as means ± SD from three independent experiments ($^{**}p < 0.01$; $^{***}p < 0.001$). (E) Knockdown of CHIP blocks the reduction of PRMT5 induced by 17-AAG treatment. HEK293T cells were transfected with siRNA Control (siCon) or siRNAs targeting CHIP (siCHIP) for 60 h, and 17-AAG was applied for another 24 h before preparing WCL for IB. (F) Knockdown of CHIP partially reverses 17-AAG-induced cell growth inhibition. HEK293T cells were transfected with siRNA Control (siCon) or siRNAs targeting CHIP (siCHIP) for 60 h, followed by the treatment with 17-AAG for another 24 h. The total cell number was counted using hemocytometer and is presented as the percentage of the control. Statistical significance ($^{*}p < 0.05$; $^{**}p < 0.01$). Representative blots from three independent experiments are shown in A, B, C, and E.

(Fig. 1B-D). Second, co-immunoprecipitation, GST pull-down, and BiFC assays demonstrated the interaction between PRMT5 and CHIP both *in vitro* and *in vivo* (Fig. 3A-D). Third, the TPR domain of CHIP was sufficient for the interaction with PRMT5 (Fig. 3E and F), which is consistent with previous reports that the TPR domain is necessary for the interaction between CHIP and its substrates [56,57]. Fourth, overexpression of CHIP dose-dependently decreased PRMT5 expression and shortened the half-life of PRMT5 (Fig. 4A, D, and E). Fifth, overexpression of CHIP, but not its mutant (K30A, H260Q), mediated PRMT5 K48-linked ubiquitination (Fig. 4F, Fig. 6B, D), whereas knockdown of CHIP blocked 17-AAG-induced ubiquitination (Fig. 5D, Fig. 6C). However, H260Q also decreased the expression level of PRMT5 when overexpressed. Contrary to previous report that H206Q brings substrates into the insoluble fraction [28,57], we did not see any significant increase of PRMT5 in the insoluble fraction (Fig. 6C). Therefore, it remains to be investigated whether CHIP may co-operate with other E3 ligases to ubiquitinate PRMT5 [58].

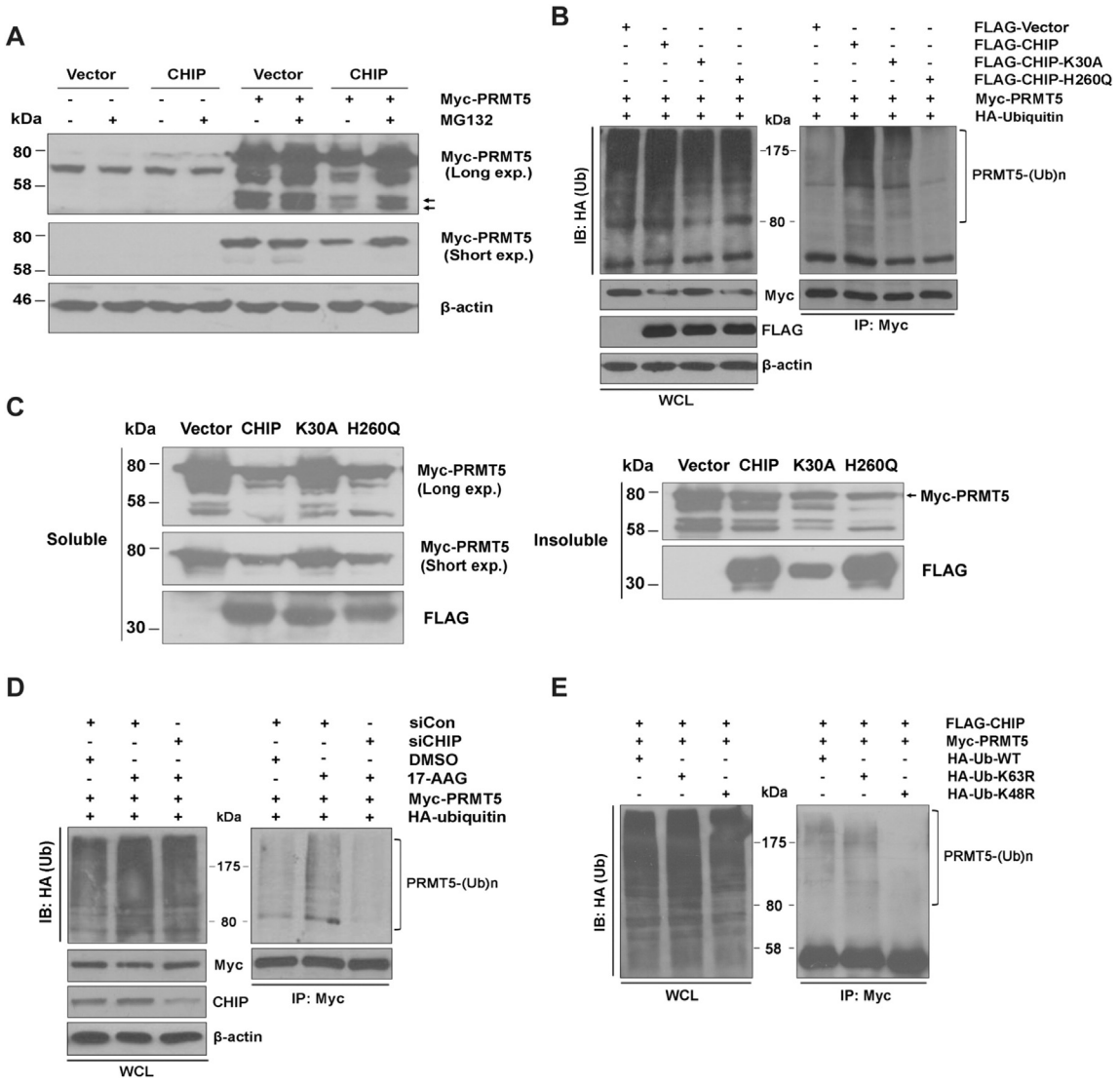

Fig. 6. CHIP promotes PRMT5 degradation through K48-linked ubiquitination. (A) MG132 inhibits the down-regulation of PRMT5 expression by CHIP. pFLAG-Vector (Vector) or pFLAG-CHIP (CHIP) was co-transfected without (−) or with pMyc-PRMT5 (+) for 48 h, and DMSO or MG132 was applied for another 6 h. Immunoblotting was performed to detect the indicated proteins. (B) CHIP, but not its mutants, promotes polyubiquitination of PRMT5. Myc-PRMT5 was co-expressed with HA-Ubiquitin, along with FLAG-Vector or FLAG-CHIP or its mutants into HEK293T cells for 48 h. Myc-PRMT5 was immunoprecipitated using anti-Myc antibody and was immunoblotted with anti-HA antibody. (C) Effect of CHIP and its mutants on the expression of PRMT5. Myc-PRMT5 was co-expressed with FLAG-CHIP and CHIP mutants (K30A and H260Q) for 48 h, and the expression level of Myc-PRMT5 (soluble and insoluble) was determined by immunoblotting. (D) Knockdown of CHIP blocks PRMT5 ubiquitination induced by 17-AAG treatment. pMyc-PRMT5 was co-transfected with pHA-Ubiquitin, along with siControl (siCon) or siCHIP for 60 h, and cells were treated with DMSO or 17-AAG for another 12 h. Whole cell lysate (WCL) was used for immunoprecipitation and was immunoblotted with anti-HA antibody. (E) CHIP mediates PRMT5 polyubiquitination through K48-linkage chain. FLAG-CHIP was co-expressed with Myc-PRMT5, along with HA-Ubiquitin-WT (HA-Ub-WT) or the indicated mutants in HEK293T cells for 48 h, and WCL was used for immunoprecipitation with anti-Myc antibody and immunoblotted with anti-HA antibody. PRMT5-(Ub)n in B, C, and D denotes the polyubiquitination of PRMT5.

CHIP-mediated client protein degradation is often coupled with the molecular chaperone system including Hsp90 and Hsp70 [21]. Hsp90 inhibitors such as GA and 17-AAG have been on clinical trials in several human cancers [23,46]. Their effects are mainly attributed by the disruption of chaperone function of Hsp90 and subsequent targeting of its client proteins for proteasomal degradation through associating with Hsp70 and E3 ubiquitin ligases [20,43,44]. Recent evidence has also shown that 17-AAG decreases PRMT5 protein expression (but not mRNA level) in ovarian cancer cell lines, suggesting that PRMT5 may be a potential client protein of Hsp90 [14,45]. We showed here that Hsp90 inhibitors GA and 17-AAG dose-dependently inhibited PRMT5 protein expression in HEK293T cells and LNCaP cells (Fig. 5A and B), and overexpression of CHIP enhanced 17-AAG-induced PRMT5 reduction and cell death (Fig. 5C and D). Given that overexpressed PRMT5 is correlated with the development and progression of several human cancers [5], our results suggest that CHIP likely mediates the inhibitory effect of 17-AAG on cancer cell growth by promoting PRMT5 polyubiquitination and degradation via the chaperone/proteasomal degradation system.

Recent reports have shown that overexpression of CHIP blocks oncogenic signaling pathways, inhibits cell migration and anchorage independent growth, and induces cell death, whereas depletion of CHIP expression increases tumor formation and metastasis in mouse models [55,59]. Interestingly, several studies have also demonstrated that the expression of CHIP in a number of cancers, such as breast cancer, gastric cancer, pancreatic cancer, and colorectal cancer [24,26], is lower than the corresponding normal tissues, and that lower expression of CHIP appears to contribute to a lower survival rate (Supplementary Fig. S2). In these cancers, CHIP actually functions as a tumor suppressor by degrading a number of important oncogenic proteins, such as hypoxia-inducible factor 1α [27], p65 [60], androgen receptor [59], c-Myc [28], EGFR [25], and histone deacetylase 6 [61]. Interestingly, PRMT5 is also

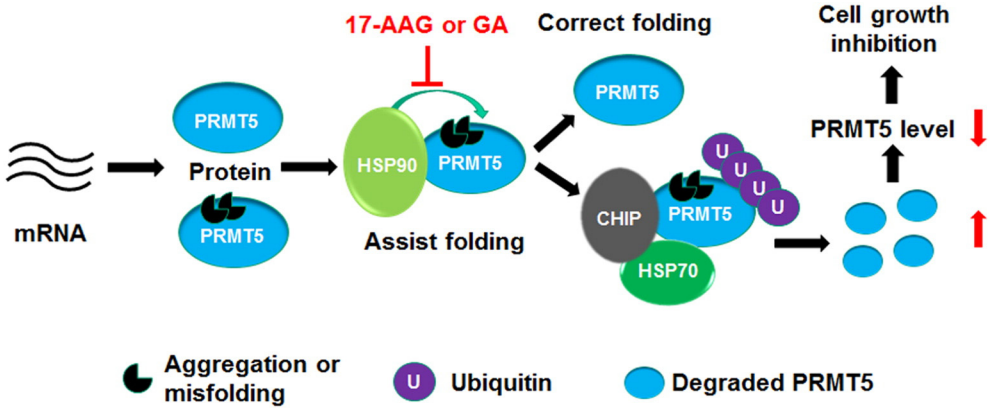

Fig. 7. The CHIP/chaperone system is involved in the regulation of PRMT5 expression. Molecular chaperone system (Hsp90 and Hsp70) plays an important role in maintaining the stability of a number of client proteins. PRMT5 is an aggregation-prone protein and is also a client protein of Hsp90. Hsp90 assists the folding of PRMT5 into a fully functional molecule (denoted as correct folding). If misfolded PRMT5 is not corrected, it will trigger the CHIP/chaperone system, thereby ubiquitinating misfolded PRMT5 for subsequent proteasomal degradation. This model is also supported by the finding that treatment of Hsp90 inhibitors, such as 17-AAG and GA, enhances CHIP-mediated ubiquitination and degradation of PRMT5.

overexpressed in these cancers. It is therefore tempting to hypothesize that the major tumor suppressor role of CHIP is through promoting the degradation of multiple oncogenic proteins such as PRMT5. In conclusion, the present study demonstrates that PRMT5 undergoes polyubiquitination and that CHIP mediates ubiquitin-dependent proteasomal degradation of PRMT5 (Fig. 7). Given that lower expression of CHIP and overexpression of PRMT5 have been observed in a number of cancers, it will be necessary to further evaluate the negative regulatory role of CHIP on PRMT5 expression in human cancer tissues.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.12.001.

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ORIGINAL ARTICLE

Protein arginine methyltransferase 5 functions as an epigenetic activator of the androgen receptor to promote prostate cancer cell growth

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Protein arginine methyltransferase 5 (PRMT5) is an emerging epigenetic enzyme that mainly represses transcription of target genes via symmetric dimethylation of arginine residues on histones H4R3, H3R8 and H2AR3. Accumulating evidence suggests that PRMT5 may function as an oncogene to drive cancer cell growth by epigenetic inactivation of several tumor suppressors. Here, we provide evidence that PRMT5 promotes prostate cancer cell growth by epigenetically activating transcription of the androgen receptor (AR) in prostate cancer cells. Knockdown of PRMT5 or inhibition of PRMT5 by a specific inhibitor reduces the expression of AR and suppresses the growth of multiple AR-positive, but not AR-negative, prostate cancer cells. Significantly, knockdown of PRMT5 in AR-positive LNCaP cells completely suppresses the growth of xenograft tumors in mice. Molecular analysis reveals that PRMT5 binds to the proximal promoter region of the AR gene and contributes mainly to the enriched symmetric dimethylation of H4R3 in the same region. Mechanistically, PRMT5 is recruited to the AR promoter by its interaction with Sp1, the major transcription factor responsible for AR transcription, and forms a complex with Brg1, an ATP-dependent chromatin remodeler, on the proximal promoter region of the AR gene. Furthermore, PRMT5 expression in prostate cancer tissues is significantly higher than that in benign prostatic hyperplasia tissues, and PRMT5 expression correlates positively with AR expression at both the protein and mRNA levels. Taken together, our results identify PRMT5 as a novel epigenetic activator of AR in prostate cancer. Given that inhibiting AR transcriptional activity or androgen synthesis remains the major mechanism of action for most existing anti-androgen agents, our findings also raise an interesting possibility that targeting PRMT5 may represent a novel approach for prostate cancer treatment by eliminating AR expression.

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INTRODUCTION

Protein arginine methyltransferase 5 (PRMT5) is a type II arginine methyltransferase that epigenetically regulates gene transcription by symmetrically dimethylating histone H4 arginine 3 (H4R3me2s), histone H3 arginine 8 (H3R8me2s) or histone H2A arginine 3 (H2AR3me2s).[1,2] PRMT5 also modulates the function of non-histone protein substrates by dimethylating arginine residues on the proteins. By regulating transcription of target genes or post-translational modifications of signaling proteins, PRMT5 is implicated in the regulation of many cellular processes such as cell cycle progression, apoptosis and DNA-damage response. Accumulating evidence shows that PRMT5 is overexpressed in several human cancers, and its expression positively correlates with disease progression and poor outcomes.[3–8] Mechanistic studies have suggested that PRMT5 may function as an oncogene by epigenetic repression of several tumor suppressor genes or by post-translational modification of signaling molecules.[9,10]

Prostate cancer remains the most common non-cutaneous cancer among American men.[11] Although many molecules and signaling pathways that regulate prostate cancer development and progression have been identified and characterized, androgen receptor (AR) signaling is the most important factor that drives prostate cancer development and progression.[12–14] Thus, targeting AR signaling, such as androgen deprivation therapy (ADT), is a standard treatment for patients with locally advanced and metastatic disease. Despite the initial response to ADT, the majority of prostate cancers progress to a lethal status known as castration resistant prostate cancer (CRPC) owing to AR reactivation, which includes AR gene amplification, AR mutations, AR splice variants, androgen-independent activation of AR by AR modulators and intratumoral *de novo* androgen synthesis in prostate cancer cells.[13,15,16] Recent evidence further shows that AR reactivation is also the major mechanism of resistance to the two next-generation anti-androgen agents abiraterone and enzalutamide.[17,18] Therefore, the expression of wild-type or mutant AR is absolutely required in both hormone naive prostate cancer and CRPC. However, compared with extensive studies of AR co-activators and co-repressors including epigenetic regulators,[19–24] how AR expression is regulated, particularly at the epigenetic level, remains largely unknown.

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Here, we report that PRMT5 is highly expressed in prostate cancer tissues and that its expression positively correlates with the expression of AR. Molecular analysis reveals that PRMT5 epigenetically activates the transcription of AR via symmetric dimethylation of H4R3 and promotes prostate cancer cell growth *in vitro* and xenograft tumor growth in mice. Given that current AR-targeting strategies, which are largely based on the inhibition of AR transcriptional activity or inhibition of androgen synthesis, are ultimately ineffective, our findings raise an interesting possibility that targeting PRMT5 may be explored as a novel therapeutic approach to inhibit or eliminate AR expression for prostate cancer treatment.

RESULTS

PRMT5 expression is required for prostate cancer cell growth in an AR-dependent manner

We and others previously reported that knockdown of PRMT5 inhibited cell growth in LNCaP cells.[25,26] To further investigate this, we examined the role of PRMT5 in DU145 and PC-3 cells by transiently knocking down PRMT5, and did not observe any significant effect on cell growth when compared with scrambled control (SC; Supplementary Figure S1a–d). Knockdown of PRMT5 in LNCaP cells also exhibited a pronounced inhibitory effect on colony formation in soft agar (Supplementary Figure S1e). Next, we established stable cell lines using LNCaP and DU145 that can be induced by doxycycline (Dox) to express short-hairpin RNA (shRNA), and confirmed that inducible knockdown of PRMT5 indeed showed significant growth inhibition in LNCaP cells (Figure 1a), but not in DU145 cells (Figure 1b). Because DU145 and PC-3 cells do not express detectable level of AR,[27] these results suggest that PRMT5 may regulate prostate cancer cell growth in an AR-dependent manner. To confirm this, we established Dox-inducible stable cell lines using LNCaP-derived CRPC cell line C4-2 cells that express a higher level of PRMT5 and AR (Supplementary Figure S2), and normal prostate epithelial RWPE-1 cells that do not express detectable AR in the absence of androgen stimulation.[28,29] Again, knockdown of PRMT5 significantly inhibited cell growth in C4-2 cells, but had no effect on cell growth in RWPE-1 cells (Figures 1c and d). Consistent with the growth inhibition in LNCaP and C4-2 cells, PRMT5 knockdown also downregulated AR expression (Figure 1e). As a result, the mRNA level of AR target genes *PSA*, *KLK2* and *TMPRSS2* was decreased by PRMT5 knockdown[30] (Figure 1f). To further confirm that AR mediates the effect of PRMT5 on the regulation of cell growth, we performed a rescue experiment by expressing FLAG-AR under the control of a CMV promoter, and observed that overexpressed FLAG-AR completely abolished the growth inhibition induced by PRMT5 knockdown (Figures 1g and h). Similar results were obtained when the LNCaP stable cell line was used and the target gene expression was partially rescued (Supplementary Figure S3). Thus, AR downregulation is likely responsible for the growth inhibition induced by PRMT5 knockdown.

Recently, a PRMT5-specific small molecule inhibitor Compound 5 (named here as BLL3.3) has been identified.[31] To determine whether inhibition of PRMT5 by BLL3.3 can recapitulate the effect of PRMT5 knockdown in prostate cancer cells, we treated LNCaP cells with BLL3.3, and observed that the growth of LNCaP cells and the expression of AR were significantly inhibited (Supplementary Figures S4a and b). No inhibitory effect was observed when DU145 and RWPE-1 cells were similarly treated with BLL3.3 (Supplementary Figures S4c and d). These results provide additional evidence that the enzymatic activity of PRMT5 is required for AR expression and cell growth in prostate cancer cells.

AR is an epigenetic target of PRMT5 in prostate cancer cells

To determine how PRMT5 regulates AR expression, we examined the effect of PRMT5 knockdown on AR transcription by performing quantitative real-time PCR (qRT-PCR), and observed that transient knockdown of PRMT5 decreased the mRNA level of AR by ~50% (Figure 2a). As PRMT5 may regulate AR transcription epigenetically or indirectly via the regulation of AR transcriptional regulators, we examined the effect of PRMT5 knockdown on the AR-Luciferase reporter gene (AR-Luc) activity, and observed that PRMT5 knockdown had no impact on the AR-Luc activity (Figure 2b). This result suggests that a native chromatin status is required for the downregulation of AR by PRMT5 knockdown. Thus it is likely through epigenetic control of AR transcription. Indeed, the symmetric dimethylation status of H4R3 was significantly enriched on the proximal promoter region of the AR gene when compared with H3R8 and H2AR3 (Figure 2c), despite that all three antibodies can efficiently immunoprecipitate histones H4, H3 and H2A (Supplementary Figure 5). Knockdown of PRMT5 exhibited a greater inhibitory effect on the methylation status of H4R3 (Figure 2d), but a lesser effect on H3R8 and H2AR3 (Supplementary Figure S6). Consistent with this, knockdown of PRMT5 reduced the binding of PRMT5 to the proximal promoter region of the AR gene (Figure 2e), and decreased the level of H4R3me2s on the AR promoter region (Figure 2f). Further, treatment of LNCaP cells with the PRMT5 inhibitor BLL3.3 also decreased the level of AR and H4R3me2s (Supplementary Figure S4b). Taken together, these results demonstrate that PRMT5 epigenetically activates AR transcription by symmetrically dimethylating H4R3.

PRMT5 interacts with Sp1 and Brg1 on the AR promoter

To determine how PRMT5 is recruited to the AR promoter, we examined whether PRMT5 interacts with Sp1, the major and only well-characterized transcription factor that positively regulates AR transcription in prostate cancer cells.[32,33] Indeed, Sp1 was co-immunoprecipitated with PRMT5 from LNCaP cells (Figure 3a). Because both H3R8me2s and H4R3me2s are associated with the activation of target gene expression when PRMT5 is associated with the ATP-dependent chromatin-remodeling enzyme Brg1,[34,35] we performed co-immunoprecipitation and found that Brg1 was also co-immunoprecipitated with PRMT5 from LNCaP cells (Figure 3b). To substantiate this finding, we established a Dox-inducible Sp1 knockdown cell line (LNCaP-shSp1) and confirmed that knockdown of Sp1 indeed repressed AR expression (Figure 3d). Significantly, knockdown of Sp1 in this cell line not only abolished the binding of Sp1 to the proximal promoter region of the AR gene (Figure 3d), but also abolished the binding of PRMT5 (Figure 3e) as well as reduced the binding of Brg1 to the same region (Figure 3f). These results together suggest that Sp1, PRMT5 and Brg1 form a complex on the AR proximal promoter region to activate AR transcription.

PRMT5 is overexpressed in human prostate cancer tissues and correlates with AR expression

Next, we examined the expression level of PRMT5 in a human prostate cancer tissue microarray (TMA) consisting of 32 benign prostatic hyperplasia (BPH) tissues and 40 prostate cancer tissues (20 with Gleason score 6 and 20 with Gleason score $\geqslant 7$), and found that PRMT5 expression was significantly higher in prostate cancer tissues than BPH tissues (Figure 4a). Although there is no statistically significant difference in the expression scores between prostate cancer tissues with Gleason score 6 and those with Gleason score 7 and above, 60% of prostate cancer tissues with Gleason score 7 and above showed moderate to high expression (total expression score 40–60) of PRMT5 whereas 40% of prostate cancer tissues Gleason score 6 had similar expression of PRMT5.

Because PRMT5 subcellular localization appears to be an important determinant of cell fate,[36,37] we compared the expression level of PRMT5 in both the cytoplasm and the nucleus and observed that some cells showed more nuclear or cytoplasmic localization of PRMT5. However, there was no significant difference in PRMT5 subcellular localization in either BPH tissues

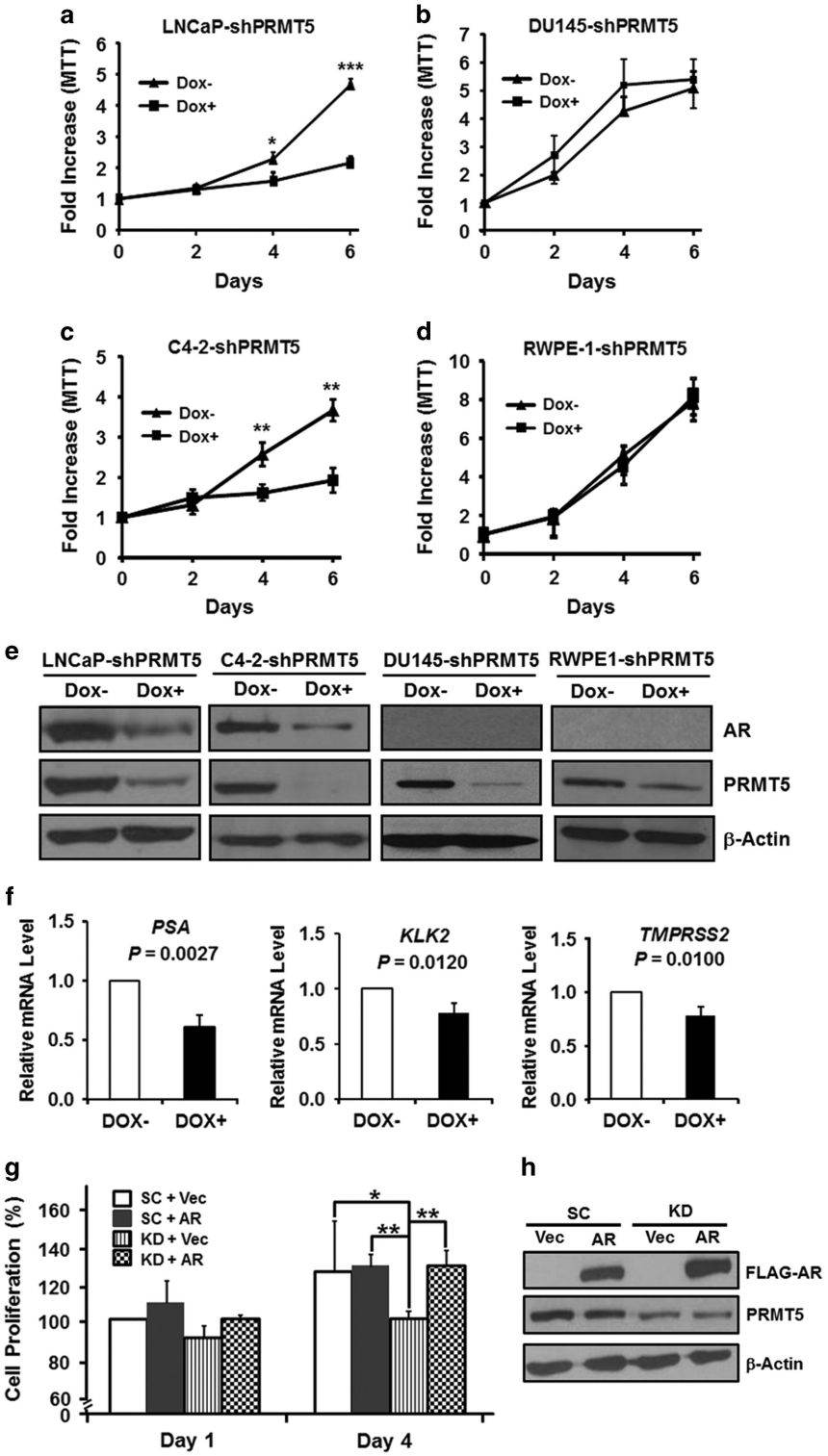

Figure 1. PRMT5 regulates prostate cancer cell growth in an AR-dependent manner. (**a**–**d**) Induction of PRMT5 knockdown by doxycycline (Dox+) inhibited cell proliferation in AR-expressing LNCaP and C4-2 cells but not in DU145 and RWPE-1 cells that do not express AR. (**e**) PRMT5 knockdown induced by Dox decreased AR expression in LNCaP and C4-2 stable cell lines. (**f**) Knockdown of PRMT5 in LNCaP-shPRMT5 cells reduced the mRNA level of the indicated AR target genes measured by qRT-PCR. (**g**) Restored cell growth by exogenous expression of FLAG-AR in LNCaP cells transiently co-transfected with SC, or pLKO-Tet-On-shPRMT5 (KD) in combination with pFLAG-CMV (Vec) or pFLAG-CMV-AR (AR). (**h**) Representative Western blots from **g** to verify the expression of FLAG-AR and the knockdown of PRMT5. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

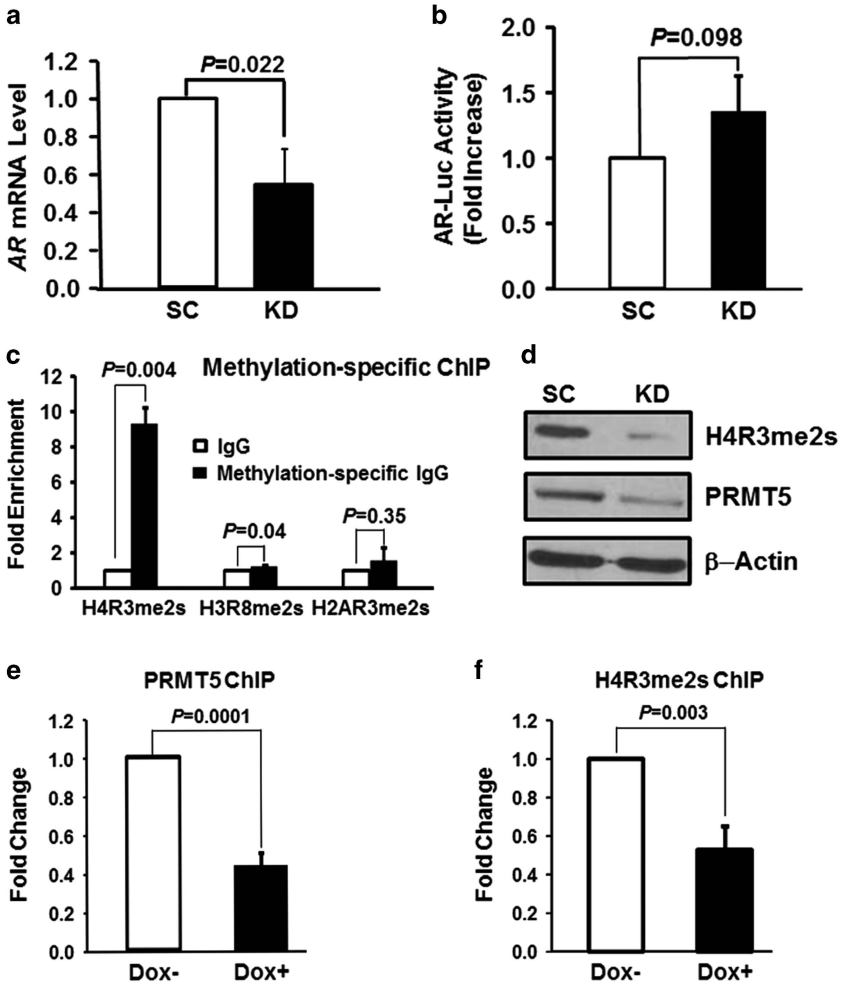

Figure 2. Epigenetic activation of AR transcription by PRMT5 in LNCaP cells. (**a**) Transient knockdown of PRMT5 (KD) reduced AR mRNA level when compared with SC. (**b**) Transient knockdown of PRMT5 had no effect on the AR-luciferase reporter gene (AR-Luc) activity. (**c**) Enrichment of H4R3me2s, but not H3R8me2s and H2AR3me2s, on the proximal promoter region of the AR gene in LNCaP cells. (**d**) Transient knockdown of PRMT5 reduced symmetric dimethylation of H4R3 (H4R3me2s). (**e**) Knockdown of PRMT5 induced by doxycycline (Dox+) reduced PRMT5 binding to the proximal promoter region of the AR gene when compared with cells without Dox (Dox –). (**f**) Knockdown of PRMT5 induced by doxycycline (Dox+) reduced the enrichment of H4R3me2s on the proximal promoter region of the AR gene when compared with cells without Dox (Dox –).

or prostate cancer tissues (Supplementary Figure S7). To analyze the correlation between AR and PRMT5 expression, we examined the expression of AR from the same TMA. In fact, PRMT5 expression in the nucleus correlated positively with AR expression in prostate tissues (Figures 4b and c). We also retrieved data from Oncomine that have >60 cases in each study, and found that PRMT5 expression correlated with AR at the transcript level in prostate cancer tissues (Figure 4d). Thus, it is likely that nuclear-localized PRMT5 may activate AR transcription in prostate tissues.

PRMT5 knockdown inhibits AR expression and suppresses the growth of xenograft tumors in mice

To determine whether PRMT5 expression is necessary for the growth of xenograft tumors in mice, we used Dox-inducible stable cell lines expressing PRMT5 shRNA (LNCaP-shPRMT5) or SC (LNCaP-SC) to establish xenograft tumors in nude mice. As shown in Figure 5a, knockdown of PRMT5 completely suppressed the growth of LNCaP xenograft tumors. In fact, tumor growth in 8 out of 10 Dox-treated mice were completely suppressed. There was no significant difference in the growth of tumors derived from LNCaP-SC regardless of the Dox status (Figure 5b). The expression level of PRMT5 and AR was also downregulated in Dox-treated residual tumor nodules derived from LNCaP-shPRMT5 when compared with Dox-untreated (Figure 5c). Similar expression of PRMT5 and AR was observed in SC control tumors regardless of the Dox status (Figure 5d). These results demonstrate that PRMT5 is required for the growth of xenograft tumors in mice.

DISCUSSION

AR signaling is a critical determinant of prostate cancer development and progression. Many studies have characterized how AR transcriptional activity is modulated by its co-activators and co-repressors.[19,21,24] However, how the transcription of AR itself is regulated, particularly at the epigenetic level, remains poorly understood. Here, we provide evidence showing that PRMT5 is a novel epigenetic activator of AR transcription in prostate cancer. First, knockdown of PRMT5 or inhibition of PRMT5 by a small molecule inhibitor specifically inhibited the growth of prostate cancer cells in an AR-dependent manner. Second, knockdown of PRMT5 specifically inhibited AR transcription. Third, PRMT5 binds to the proximal promoter region of the AR gene along with Sp1

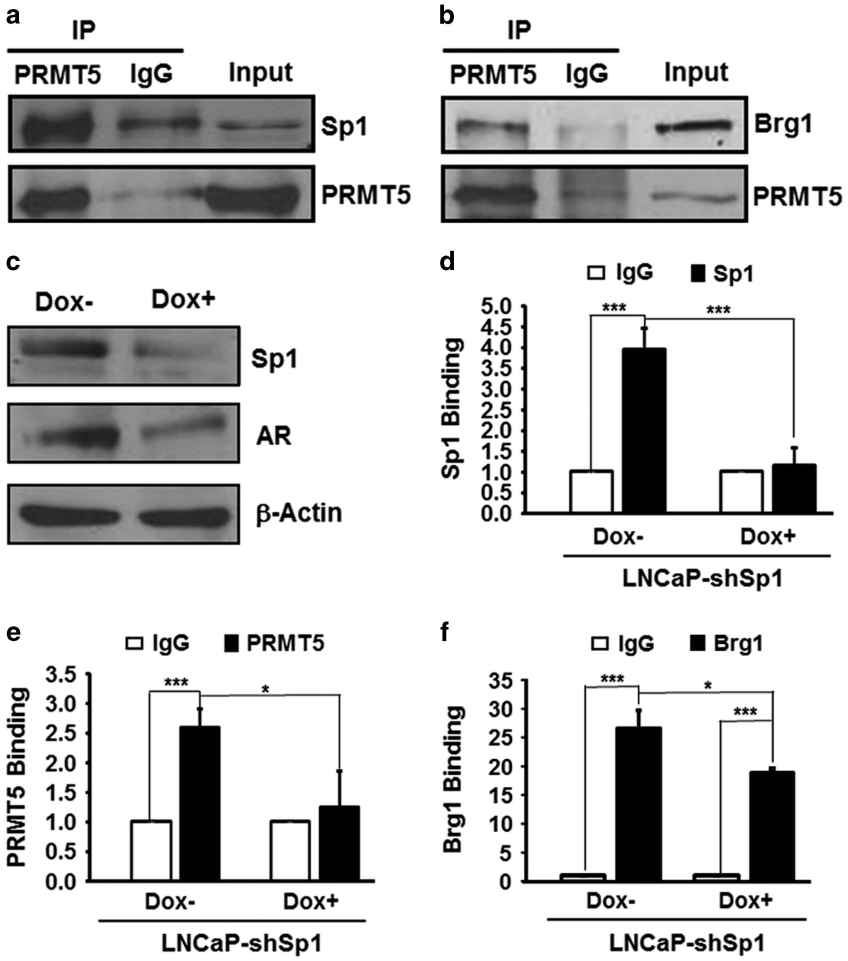

Figure 3. PRMT5 interacts with Sp1 and Brg1 on the proximal promoter region of the AR gene in LNCaP cells. (**a**) Co-immunoprecipitation of Sp1 with PRMT5. (**b**) Co-immunoprecipitation of Brg1 with PRMT5. (**c**) Knockdown of Sp1 induced by doxycycline (Dox+) reduced AR expression in Dox-inducible stable cell line LNCaP-shSp1. (**d**–**f**) Dox-induced knockdown of Sp1 reduced the binding of Sp1, PRMT5 and Brg1 to the same proximal promoter region of the AR gene. $*P < 0.05$ and $***P < 0.001$.

and Brg1. Fourth, H4R3me2s is highly enriched on the proximal promoter region of the AR gene. Fifth, PRMT5 is highly expressed in prostate cancer tissues and its expression correlates positively with AR expression at both mRNA and protein levels. Finally, depletion of PRMT5 expression completely suppressed the growth of LNCaP xenograft tumors in mice by downregulating AR expression.

Transcriptional regulation of gene expression is a tightly regulated process that involves the participation of multiple transcriptional regulatory proteins such as transcription factors, co-activators and co-repressors as well as chromatin-remodeling enzymes. Consistent with the fact that Sp1 is the major and well-characterized transcription factor that activates AR transcription in prostate cancer cells,[33,38] we indeed confirmed that Sp1 binds to the AR promoter and regulates AR expression in LNCaP cells. Because PRMT5 interacts with Sp1 and Brg1 and because Sp1 knockdown also reduces the binding of PRMT5 to the AR promoter, we suggest that Sp1 may recruit PRMT5 to the AR promoter. Interestingly, Brg1, an ATP-dependent chromatin remodeler,[39] was also recruited to the AR promoter through its interaction with PRMT5. This finding suggests that PRMT5-mediated H4R3 dimethylation could also activate transcription of target genes such as AR when Brg1 is recruited to the promoters (Figure 6), though PRMT5 generally represses transcription of target genes. Interestingly, PRMT5-mediated H3R8 dimethylation is also involved in transcriptional activation of target genes when Brg1 is recruited to the target gene promoters.[34,35] Although this manuscript was in preparation, a recent report showed that PRMT5 can dimethylate H4R3 and H3R8 to regulate the expression of the protein kinase FLT3 in acute myeloid leukemia cells via two distinct pathways.[40] Thus, dimethylation of either H3R8 or H4R3 by PRMT5 may permit ATP-dependent chromatin remodeling, leading to activation or repression of target gene transcription. Given that PRMT5 and Brg1 also cooperate to repress transcription of target genes[41–43] and that AR transcription is subjected to the regulation of DNA methylation and histone lysine methylation,[44] it is likely that AR transcription is subjected to a high order of epigenetic regulation. Future studies to gain insight into the epigenetic regulation of AR may offer new opportunities to develop novel targeting strategies to inhibit or even eliminate AR expression. Because PRMT5 may exhibit an opposite role in the cytoplasm and nucleus in cells,[9,25] it remains to be determined whether cytoplasmic- and nuclear-localized PRMT5 may have distinct effects on the transcription of AR.

The present finding has significant clinical implications due to the central role of AR in prostate cancer development and progression. Our findings here, together with a previous study showing that PRMT5 may form a complex with MEP50 and AR to modulate the transcriptional activity of AR,[45] raise an interesting

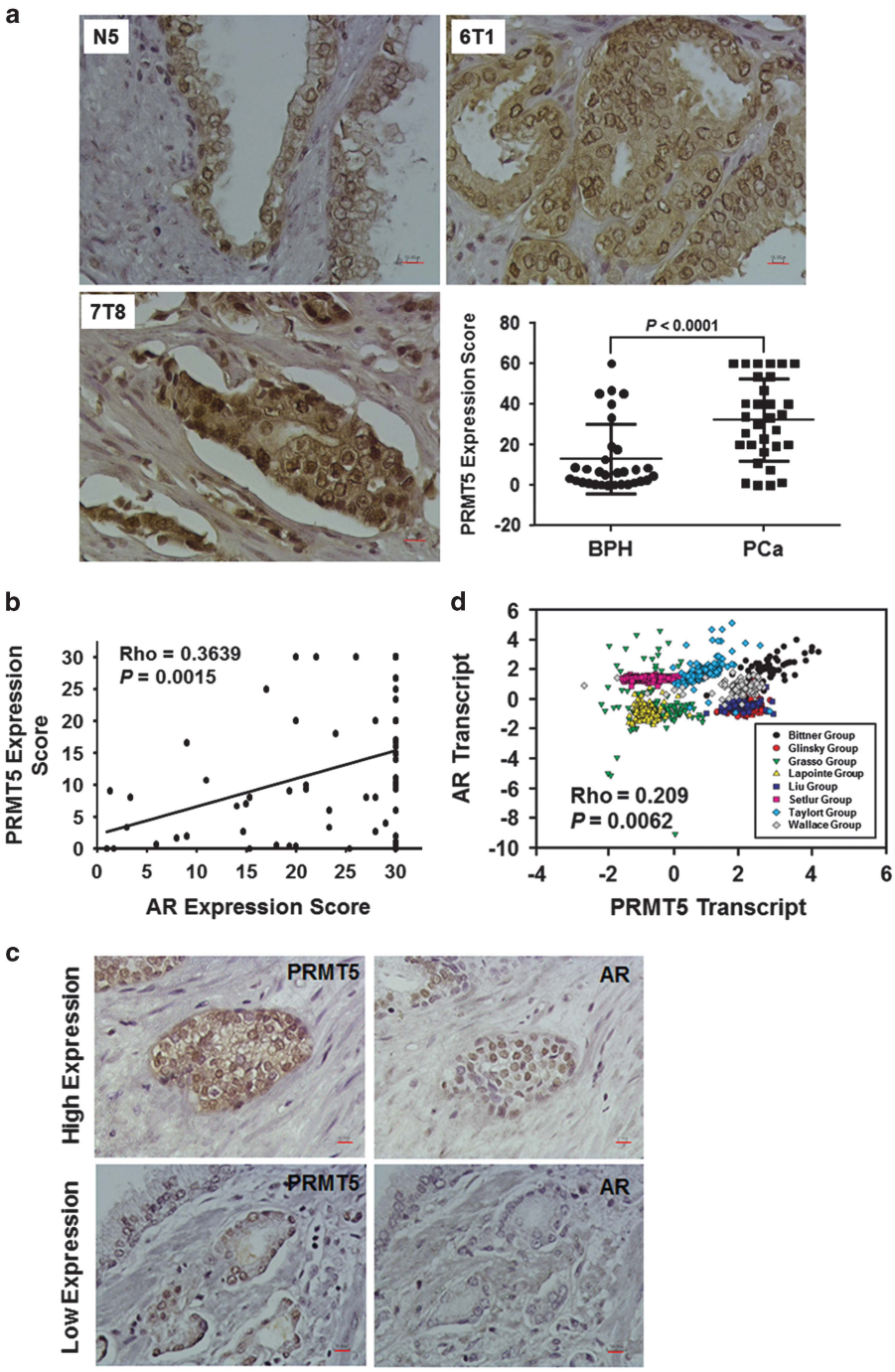

Figure 4. PRMT5 expression correlates positively with AR expression in prostate cancer. (**a**) Shown are representative immunohistochemistry staining images (magnification ×400) of PRMT5 in benign tissue (N5), Gleason 6 prostate cancer tissue (6T1) and Gleason 7 prostate cancer tissue (7T8). The total expression score of PRMT5 is significantly higher in prostate cancer tissues (PCa) when compared with BPH. Scale bar, 30 μm. (**b**) PRMT5 expression correlates positively with AR expression at the protein level in the same TMA from **a**. (**c**) Representative images of PRMT5 and AR expression from serial sections of prostate cancer tissues. The upper panels show higher expression of both PRMT5 and AR in the nucleus and the lower panels show weaker expression of both PRMT5 and AR in the nucleus. Scale bar, 30 μm. (**d**) PRMT5 expression correlates positively with AR expression at the transcript level. The data were retrieved from Oncomine database.

possibility that targeting PRMT5 may have a dual effect on both the expression and activity of AR. Thus, PRMT5 may be an ideal target for development of novel therapeutics. As radiotherapy in combination with adjuvant ADT is the current standard treatment for locally advanced prostate cancer, combining radiotherapy with PRMT5 targeting may be an alternative approach. Perhaps targeting AR expression by inhibiting PRMT5 may avoid some adverse effects often seen with ADT. It is worth noting that PRMT5 also regulates the expression of AR in the CRPC line C4-2. As AR reactivation is the major mechanism underlying the development of CRPC[13,14] and the resistance to the next-generation anti-androgen therapy,[17,18] targeting PRMT5 alone or in combination

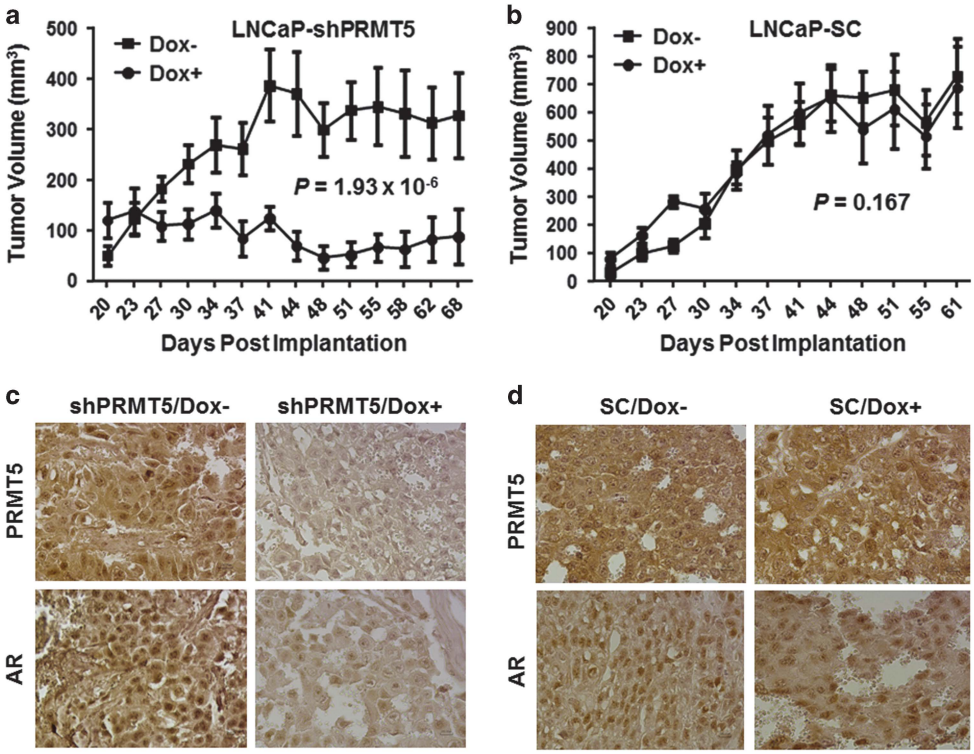

Figure 5. Knockdown of PRMT5 suppresses the growth of xenograft tumors in mice. (**a**) LNCaP-shPRMT5 cells were implanted subcutaneously into the right lower flanks of 10 nude mice per group, and the tumor growth was monitored twice weekly in Dox-treated (Dox+) and untreated (Dox−) mice. (**b**) Similar experiment was performed as described in **a** for LNCaP-SC cell line. (**c** and **d**) Representative images showing inhibition of PRMT5 and AR expression in Dox-treated tumor nodules. No effect on PRMT5 and AR expression in xenograft tumors derived from LNCaP-SC was observed. Scale bar: 10 μm.

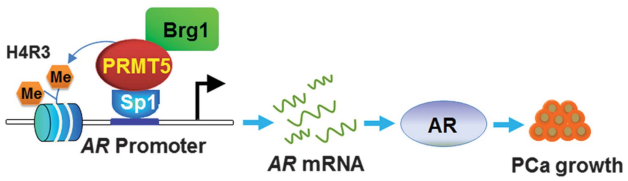

Figure 6. Proposed model for epigenetic activation of AR transcription by PRMT5.

with other AR-targeting agents may exhibit a better treatment efficacy than the existing treatments. Given that two small molecule inhibitors of PRMT5 have been developed,[31,40,46] preclinical evaluation of these inhibitors alone or in combination with radiotherapy or other AR-targeting agents may lead to the development of novel therapeutic approaches for prostate cancer treatment.

MATERIALS AND METHODS

Cell lines and culture

Prostate cancer cell lines LNCaP, DU145, and PC-3 as well as RWPE-1 cells were purchased from ATCC (Manassas, VA, USA) and C4-2 cells were purchased from M.D. Anderson Cancer Center (Houston, TX, USA). All frozen stock received were immediately expanded and aliquots were prepared and stored in liquid nitrogen for future use, and cells were maintained for no longer than 3 months as described previously.[30,47] Cell line authentication was performed by IDEXX BioResearch (IMPACT I). The establishment of stable cell lines was described previously.[26,30]

Plasmid construction

The pLKO-Tet-On plasmid for expressing shRNA was obtained from Addgene (Cambridge, MA, USA),[48] and the two shRNA sequences that target 5′-GCCCAGTTTGAGATGCCTTAT-3′ (#1577) and 5′-CCCATCCTCTTCCCTATTAAG-3′ (#1832) for PRMT5 knockdown and that target 5′-CCACTCCTTCAGCCCTTATTA-3′ (#2310) for Sp1 knockdown were selected for constructing pLKO-Tet-On-shPRMT5 and pLKO-Tet-On-shSp1 as described previously.[30] The pLKO-Tet-On-SC and pFLAG-CMV-AR were constructed before.[30] The *AR* promoter luciferase reporter gene construct and the *PSA* promoter luciferase reporter gene construct were kindly provided by Dr Donald Tindall. pFLAG-CMV-AR was made by subcloning the *AR* cDNA into pFLAG-CMV vector. All plasmids were confirmed by DNA sequencing.

Cell proliferation assay

The cell proliferation assay was performed using MTT reagent (Sigma, St Louis, MO, USA). For transient transfection experiments, LNCaP, DU145 or PC-3 cells (4×10^3) were seeded in 48-well plates for 24 h, and then transiently transfected with pLKO-Tet-On-shPRMT5 (#1577) or the SC control using FuGENE HD or FuGENE 6 (Promega, Madison, WI, USA) for 96 h after the transfection. For MTT analysis, cell medium was removed and 70 μl of MTT solution (0.5 mg/ml) was added into each well and incubated at 37 °C for 4 h. At the end of incubation, MTT solution was removed and 130 μl of DMSO was added into each well and incubated at 37 °C for another 10 min. The plates were then read at 560 nm with TECAN Microplate Reader (TECAN, Mannedorf, Switzerland). For LNCaP, DU145, C4-2 and RWPE-1 stable cell lines, similar procedure was followed except that Dox was added at 1 μg/ml to induce PRMT5 knockdown during culture. At least three independent experiments were performed and the mean ± s.d. was presented. Student's *t*-test was performed to determine the statistical significance. The effect of PRMT5 inhibitor BLL3.3 on the growth of LNCaP, DU145 and RWPE-1 cells was similarly determined by MTT.

Soft-agar growth assay

The soft-agar growth assay to measure anchorage-independent proliferation of LNCaP cells was performed by using the 96-well plate format as described previously.[49] Briefly, LNCaP cells were transfected with pLKO-Tet-On-shPRMT5 (#1577) or pLKO-Tet-On-SC for 24 h, and then 2.5×10^3 cells were added into the middle layer agar. Dox was added into each layer

of soft agar at 1 μg/ml to induce the expression of shRNAs. The plates were incubated at 37 °C, 5% CO_2 for 7 days. To quantify the colony-formation efficiency, 16 μl of AlamerBlue Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) was added into each well and incubated at 37 °C for another 4 h. Fluorescence intensity was measured at 570EX nm/600EM nm using Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Experiments were performed in triplicate, and results from three independent experiments were analyzed and presented as mean ± s.d. Student's *t*-test was used to determine the statistical significance.

qRT-PCR and western blotting

To determine the effect of PRMT5 knockdown on AR expression, PRMT5 were transiently or stably knocked down in LNCaP cells for 96 h, and total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of total RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Promega) according to manufacturer's instruction. The qRT-PCR analysis of *AR* or AR target genes (*PSA*, *KLK2*, *TMPRSS2*) was performed as described previously.[30] Antibodies against AR (SC-816, Santa Cruz, CA, USA), PRMT5 (07-405, Millipore, Billerica, MA, USA), PSA (1984-1, Epitomics, Burlingame, CA, USA), FLAG (Sigma, F-1804), Sp1 (ab13370, Abcam, Cambridge, MA, USA), H4R3me2s (Abcam, ab5823), H3R8me2s (Abcam, ab130740), H2AR3me2s (Abcam, ab22397), and Brg1 (Abcam, ab110641) were used for western blotting analysis.

Chromatin immunoprecipitation assay

The LNCaP stable cell line or parental cells were cultured in the presence or absence of Dox (1 μg/ml) for 96 h. At the end of induction, 270 μl of 37% formaldehyde was added into each dish and incubated at room temperature for 10 min. Then 1 ml of 1.25 M glycine was added to stop the cross-linking reaction. Cells were then harvested, resuspended in 1 ml of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 0.5% NP-40, 0.5 mM DTT, and protease and phosphatase inhibitors), and finally sonicated (Branson Sonifier250set, Wilmington, NC, USA) to prepare sheared chromatin. Antibodies against PRMT5 (Millipore, 07-405), Sp1 (Santa Cruz, SC7824), Brg1 (Abcam, ab110641), H4R3me2s (Abcam, ab5823), H3R8me2s (Abcam, ab130740), H2AR3me2s (Abcam, ab22397) and IgG (Santa Cruz, SC2027) were used to immunoprecipiate protein-DNA complexes for isolation of PCR-ready DNA using the Fast ChIP protocol described previously.[50] The co-immunoprecipitated proximal promoter region of *AR* (−493 to −226) was quantified by qRT-PCR. Results were normalized to the IgG control and are presented as mean ± s.d. from three independent experiments. Student's *t*-test was used to determine the statistical significance.

Co-immunoprecipitation of PRMT5 with Sp1 and Brg1

Total cell lysate of LNCaP cells was prepared in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 0.5% NP-40, 0.5 mM DTT, and protease and phosphatase inhibitors) for co-immunoprecipitation. Anti-PRMT5 antibody or IgG was used to immunoprecipitate PRMT5 from 500 μg of total lysate, and co-immunoprecipitated Sp1 and Brg1 was detected with Sp1 and Brg1 antibodies.

Expression of PRMT5 and AR and the analysis of their correlation in prostate cancer tissues

A TMA consisting of 32 BPH tissues and 40 prostate cancer tissues (20 with Gleason score 6 and 20 with Gleason score ⩾ 7) was used for immunohistochemistry analysis of PRMT5 and AR expression. Briefly, paraffin section of the TMA was deparaffinized in xylene and rehydrated in graded ethanol, followed by inactivation of endogenous peroxidase activity in 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by heating slides in 10 mM Tris-HCl (pH 10) for 30 min in microwave. After three washes with phosphate-buffered saline containing 0.1% Tween 20 (PBST), slides were blocked in 5% non-fat milk in PBST at room temperature for 1 h. The primary antibodies against PRMT5 or AR was incubated at 4 °C overnight, followed by three washes with PBST and incubation with HRP-conjugated anti-rabbit secondary antibodies (Amersham, Pittsburgh, PA, USA) at room temperature for 1 h. The signal was developed with diaminobenzidine for 10 min, and sections were counterstained with hematoxyline. The semi-quantification of PRMT5 and AR expression was performed as described previously with slight modifications.[51] The intensity was scored as 0 (no expression), 1 (low expression), 2 (moderate expression) and 3 (high expression), and the percentage of cells showing the expression was scored ranging from 0 to 10 with 10 as the highest percentage (100%). The expression score for cytoplasmic- and nuclear-localized PRMT5 was respectively determined by the intensity score times the percentage (0–30), and the total expression score is the sum of the cytoplasmic and nuclear expression scores (0–60). The unpaired *t*-test was used to determine the statistical significance of the total mean expression score between BPH and prostate cancer tissues, and paired *t*-test was used to determine the difference in expression scores between cytoplasmic-localized PRMT5 and nuclear-localized PRMT5. The same semi-quantification method was used for AR expression in the nucleus.

To determine the correlation between the expression of PRMT5 and AR in the nucleus in prostate tissues, their nuclear expression scores were used for Pearson's analysis. To determine the correlation of PRMT5 and AR expression at the transcript level, we retrieved their expression data from 8 studies that have >60 tissues from Oncomine. The statistic Q was calculated to test the homogeneity of effect sizes across studies for each of the three methods (Pearson's, Spearman's and Kendall's),[52] and it was found that the effect sizes across studies were not homogeneous (all with *P*-value < 1e − 12). Therefore, we used a random-effects model for the meta-analysis of each method.[53]

Xenograft tumor growth in nude mice

Animal experiments were approved by the Purdue University Animal Care and Use Committee. Male athymic nude mice (5–7 week old) were purchased from Harlan Laboratories (Indianapolis, IN, USA), and 3×10^6 cells of established stable cell lines that inducibly express PRMT5 shRNA or SC were co-injected subcutaneously into the right lower flank of 20 mice with Matrigel (1:1 in volume). Assuming that PRMT5 knockdown can reduce tumor volume by 30% and that standard deviation within each group is about 25% of the mean tumor volume, a sample size of 10 male mice per group will have over 80% power to detect a 30% difference between the two groups at alpha level 0.05. Mice were randomly divided into two groups (10 mice/group) for each stable cell line by using Excel-based randomization method, and treated with Dox (1 mg/ml in drinking water) or without Dox (drinking water only). Tumor growth was monitored twice weekly, and tumor volume was calculated using $\frac{1}{2} \times L \times W \times H$ without using blinding method. At the end of experiments, tumors were resected and formalin fixed, and paraffin embedded. Immunohistochemistry analysis of PRMT5 and AR expression was similarly performed as described above. We used the following linear mixed model to model the j-th observed xenograft tumor volume of i-th mouse, that is, yij, assuming cubic polynomial growth of tumors over time,

$$y_{ij} = \gamma_{i0} + \gamma_{i1} t_j + \gamma_{i2} t_j^2 + \gamma_{i3} t_j^3 + \varepsilon_{ij}, \;\; \varepsilon_{ij} \sim N(0, \sigma^2), \;\; \gamma_{ik} \sim N(\beta_k + \delta_k D_i, \sigma_k^2),$$

Where, t_j is the number of days after implantation for the j-th observation, D_i indicates whether the i-th subject is under Dox. The random-effects are independent, and the errors of the same subject are assumed to follow a first-order continuous autoregressive model.

To evaluate the effects of Dox on the tumor growth, we are subject to test the H_0: $\delta_0 = \delta_1 = \delta_2 = \delta_3 = 0$ against H_a: at least one of δ_0, δ_1, δ_2, δ_3 is not zero.

We used the likelihood ratio test (χ^2-test) to conduct the hypothesis tests.

For PRMT5 knockdown, the *P*-value is 1.9305×10^{-6}. For SC, the *P*-value is 0.1670. Error bar, s.e.m.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Legends to Supplementary Figures

Figure S1. Transient knockdown of PRMT5 inhibits prostate cancer cell growth in LNCaP cells but not DU145 and PC-3 cells. (**a**) Knockdown of PRMT5 in the indicated prostate cancer cells transiently transfected with scrambled control (SC) or pLKO-Tet-On-shPRMT5(#1577). (**b-d**) Transient knockdown of PRMT5 inhibited cell proliferation in LNCaP, but not in DU145 and PC-3 cells assayed by MTT. (**e**) Transient knockdown of PRMT5 by pLKO-Tet-On-shPRMT5(#1577) significantly inhibited colony formation in soft agar assay when compared with SC.

Figure S2. Western blotting analysis of PRMT5 and AR expression in LNCaP and C4-2 cells. Higher expression level of PRMT5 and AR was observed in C4-2 cells.

Figure S3. Rescue of PRMT5 knockdown-induced growth inhibition by AR overexpression in LNCaP cells. (**a**) LNCaP-shRNA stable cell line were transfected with pFLAG (Vector) or pFLAG-AR (AR) and cultured for 7 days in the presence of doxycycline (Dox+) or absence of doxycycline (Dox-). Cell growth was determined by Trypan blue staining, and the fold increase of cell growth was determined by dividing the total number of cells at day 7 by the number of cells seeded initially. (**b**) Cell lysate from (**a**) was used to determine the total expression of AR using an anti-AR antibody and the expression of PRMT5 using an anti-PRMT5 antibody. (**c-e**) Similar experiments were performed as described in (**a**), and total RNA was isolated for qPCR quantification of *PSA*, *TMPRSS2* and *KLK2*. Three independent experiments were performed and mean+SD was presented. The Student's *t*-test was used for *P* value calculation between the indicated two groups.

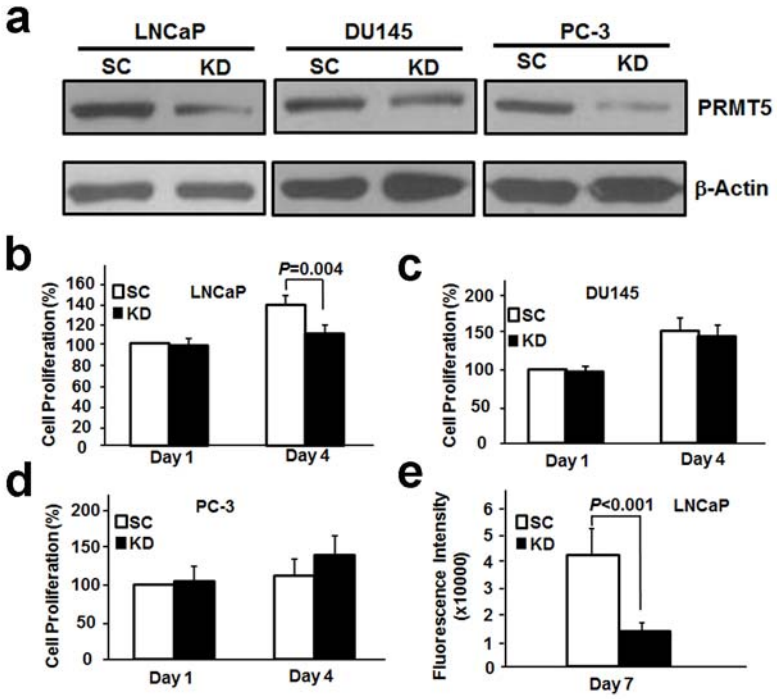
Figure S4. **Inhibition of PRMT5 by a small molecule inhibitor attenuates cell proliferation and reduces AR expression in LNCaP cells**. (**a**) LNCaP cells were treated with 10 μM of BLL3.3, a selective small molecule inhibitor of PRMT5, and cell growth was determined by MTT. (**b**) LNCaP cells were incubated with BLL3.3 (10 μM) for 6 days, and the down-regulation of AR expression and the inhibition of symmetric dimethylation of H4R3 (H4R3me2) by the inhibitor were confirmed by Western blotting. Note that BLL3.3 had no effect on the expression level of PRMT5. (**c** and **d**) Similar cell growth experiments were performed for DU145 and RWPE-1 as LNCaP and no inhibitory effect was observed.

Figure S5. **Immunoprecipitation of histones H4R3, H3R8 and H2A by methylation-specific antibodies**. LNCaP cells were crosslinked and chromatins were fragmented as did for ChIP analysis except that proteins were not digested with protease K. Antibodies that recognize H4R3me2s, H3R8me2s and H2AR3me2s were used to immunoprecipitate H4R3, H3R8 and H2A, respectively. All three histones were efficiently immunoprecipitated when compared with the IgG control.

Figure S6. **Effect of PRMT5 knockdown on the methylation status of histones.** The established doxycycline (Dox)-inducible PRMT5 knockdown cell line LNCaP-shPRMT5 was induced by Dox (1 μg/ml) for 96 h (Dox+) or without Dox induction (Dox-), and total cell lysate was prepared for Western blotting analysis of H4R3me2s, H3R8me2s, and H2AR3me2s.

Figure S7. **Expression of PRMT5 in the cytoplasm and nucleus in prostate tissues.** The expression score of both cytoplasmic and nuclear expression of PRMT5 in a prostate cancer TMA was semi-quantified, and the paired *t*-test was used to determine the statistical significance in the subcellular localization of PRMT5 in both BPH (32 cases), prostate cancer tissues with Gleason score 6 (20 cases), and prostate cancer tissues with Gleason score 7 and above (20 cases).

Figure S1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LNCaP
DU145
PC-3
SC
KD
SC
KD
SC
KD
PRMT5
β-Actin
b
LNCaP
P=0.004
SC
KD
Cell Proliferation (%)
160
140
120
100
80
60
40
20
0
Day 1
Day 4
c
DU145
SC
KD
Cell Proliferation (%)
200
150
100
50
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Day 1
Day 4
d
PC-3
SC
KD
Cell Proliferation (%)
200
150
100
50
0
Day 1
Day 4
e
P<0.001
LNCaP
SC
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Fluorescence Intensity (x10000)
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3
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1
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Figure S2

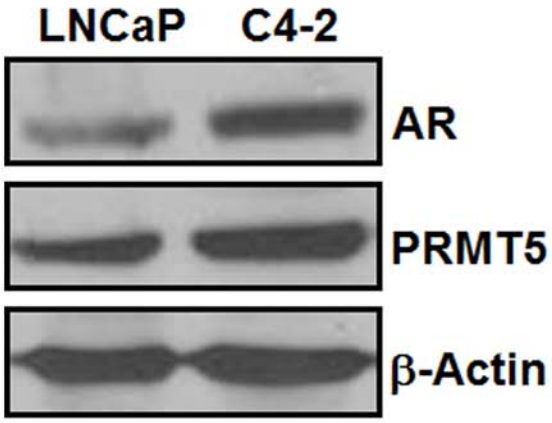
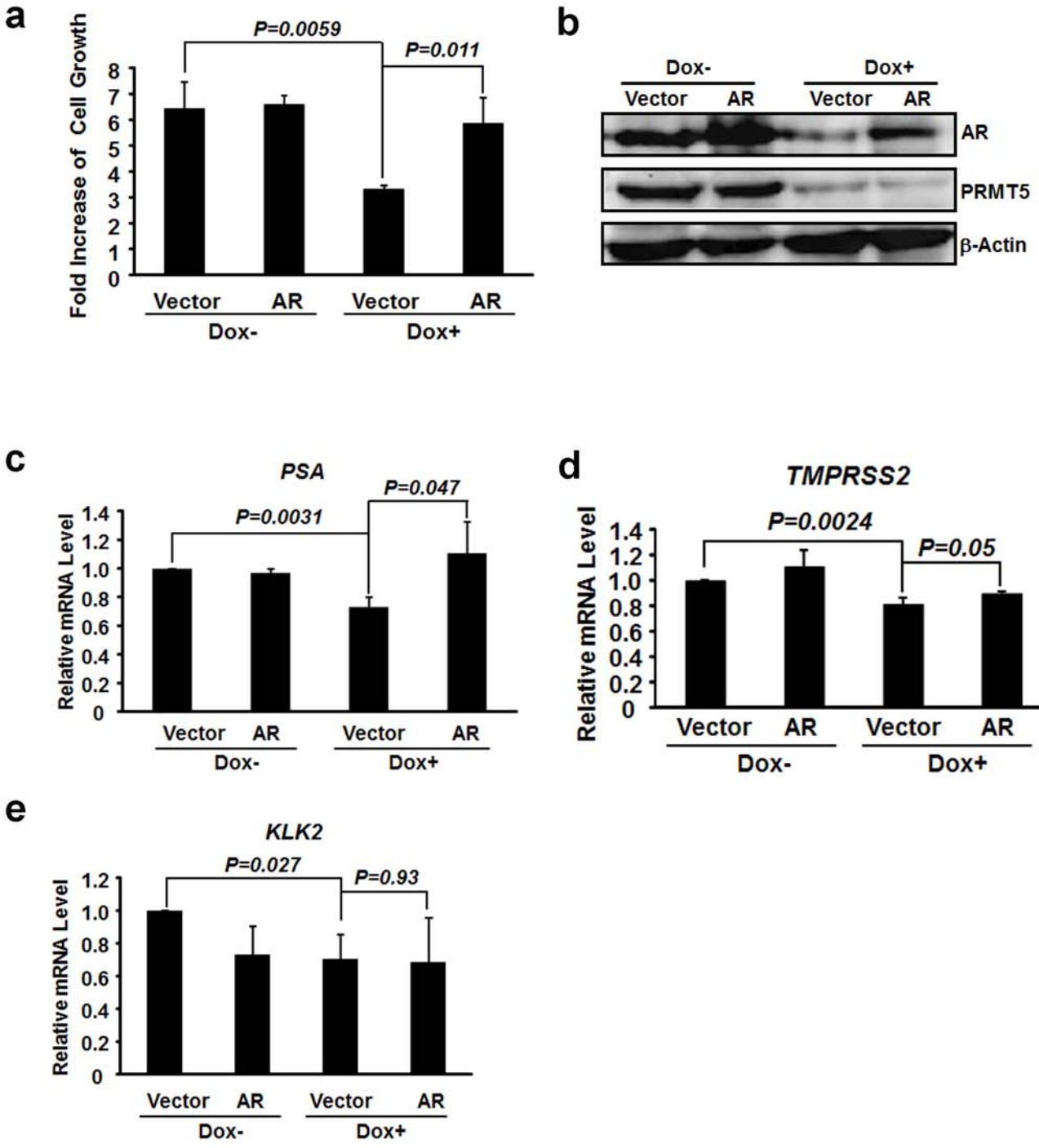


Figure S3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Fold Increase of Cell Growth
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7
8
Vector
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Dox-
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b
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Dox+
Vector
AR
Vector
AR
AR
PRMT5
β-Actin
c
PSA
P=0.0031
P=0.047
Relative mRNA Level
0
0.2
0.4
0.6
0.8
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1.2
1.4
Vector
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Vector
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Dox-
Dox+
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AR
Vector
AR
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Dox+
e
KLK2
P=0.027
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Relative mRNA Level
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Vector
AR
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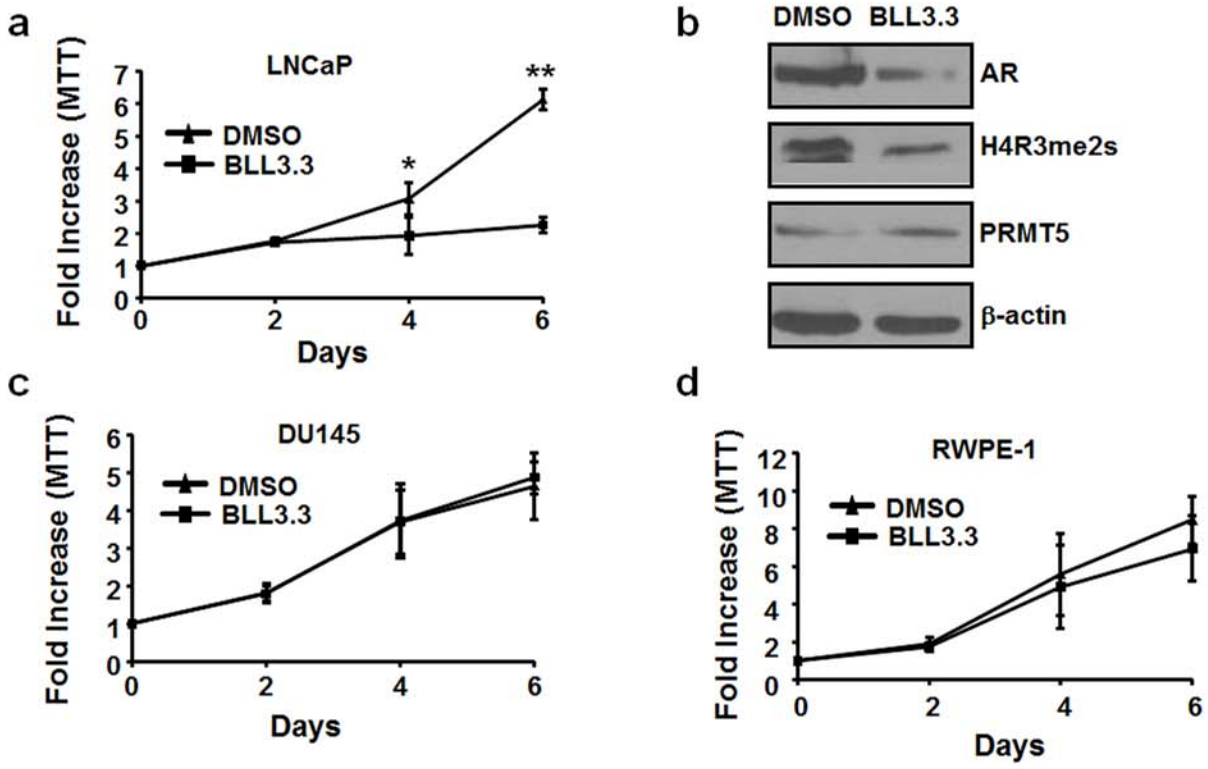
Dox-
Dox+

Figure S4


a
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Fold Increase (MTT)
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**
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b
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H4R3me2s
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c
DU145
Fold Increase (MTT)
DMSO
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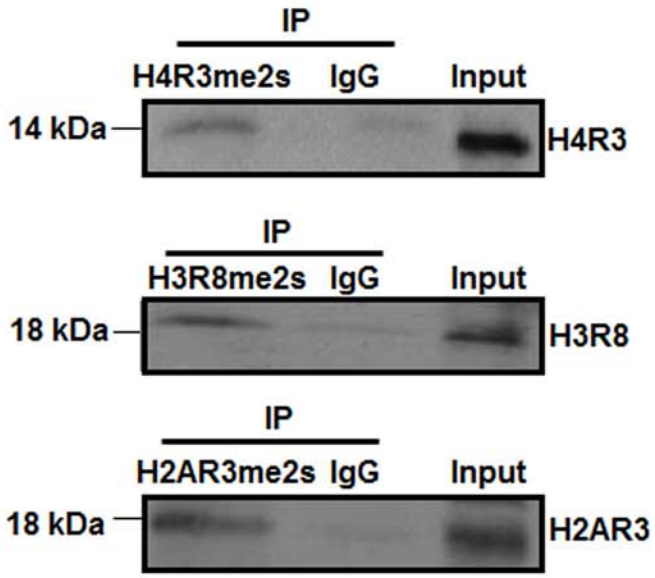
Days
d
RWPE-1
Fold Increase (MTT)
DMSO
BLL3.3
Days

Figure S5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IgG
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IP
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18 kDa
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H2AR3me2s
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Input
18 kDa
H2AR3

Figure S6

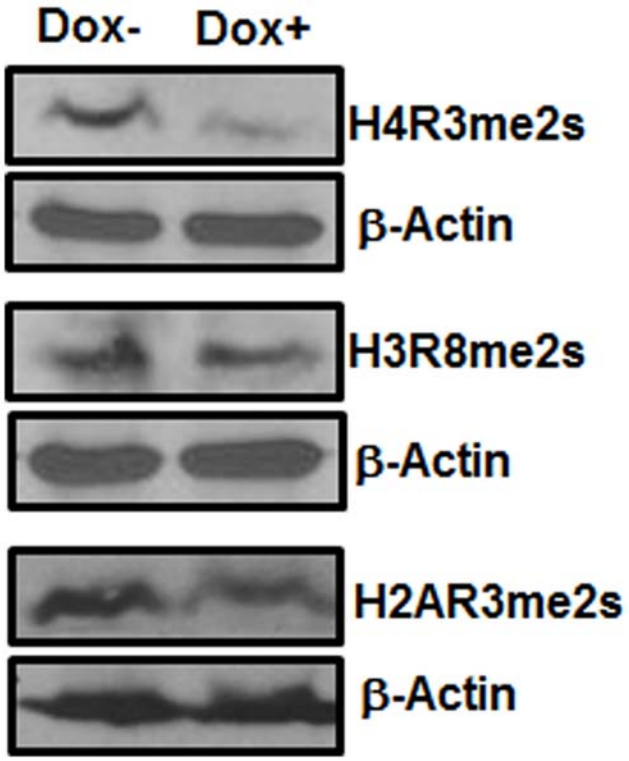
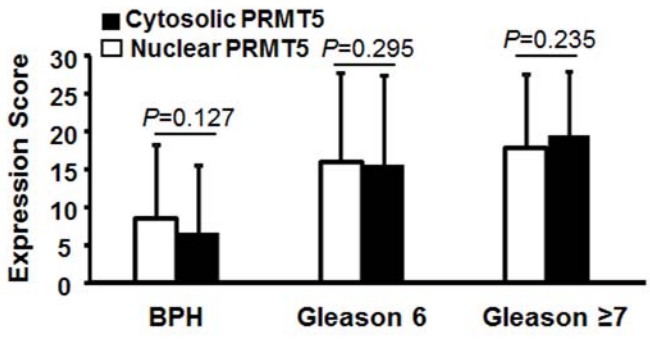


Figure S7


Cytosolic PRMT5
Nuclear PRMT5
Expression Score
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25
20
15
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5
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P=0.127
P=0.295
P=0.235
BPH
Gleason 6
Gleason ≥7

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REVIEW ARTICLE
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Neuroendocrine differentiation in prostate cancer: a mechanism of radioresistance and treatment failure

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Neuroendocrine differentiation (NED) in prostate cancer is a well-recognized phenotypic change by which prostate cancer cells transdifferentiate into neuroendocrine-like (NE-like) cells. NE-like cells lack the expression of androgen receptor and prostate specific antigen, and are resistant to treatments. In addition, NE-like cells secrete peptide hormones and growth factors to support the growth of surrounding tumor cells in a paracrine manner. Accumulated evidence has suggested that NED is associated with disease progression and poor prognosis. The importance of NED in prostate cancer progression and therapeutic response is further supported by the fact that therapeutic agents, including androgen-deprivation therapy, chemotherapeutic agents, and radiotherapy, also induce NED. We will review the work supporting the overall hypothesis that therapy-induced NED is a mechanism of resistance to treatments, as well as discuss the relationship between therapy-induced NED and therapy-induced senescence, epithelial-to-mesenchymal transition, and cancer stem cells. Furthermore, we will use radiation-induced NED as a model to explore several NED-based targeting strategies for development of novel therapeutics. Finally, we propose future studies that will specifically address therapy-induced NED in the hope that a better treatment regimen for prostate cancer can be developed.

Keywords: neuroendocrine differentiation, prostate cancer, CREB, ATF2, radiosensitization, radiotherapy, cancer stem cell, EMT

INTRODUCTION

Prostate cancer is the second leading cause of cancer death among men in developed countries (1). In 2015, it is estimated that 27,540 men will die from prostate cancer in US according to American Cancer Society. Most of these deaths are due to the progression of localized diseases into metastatic, castration-resistant, prostate cancer (CRPC).

Based on prostate specific antigen (PSA) level, tumor grade, and the extent of primary tumor in the prostate gland, clinically localized prostate cancer is classified into low-risk (PSA $\leq$10 ng/ml, Gleason score $\leq$6, and stage T1c–T2a), intermediate-risk (PSA $>$10 but $\leq$20 ng/ml, Gleason score 7, or stage T2b), and high-risk (PSA $>$20, Gleason score $\geq$8, or stage T2c) (2, 3). While a majority of low-risk disease is cured with surgery or radiotherapy (RT), intermediate- and high-risk disease has a relatively high rate of recurrence following a definitive therapy. For example, approximately 30–50% of high-risk, clinically localized, prostate cancer treated with RT develop a biochemical recurrence within 5 years post-therapy, and about 20% die of prostate cancer within 10 years (4–7). Given that about 25% of patients are diagnosed with a high-risk disease at presentation (8), there has been a major effort to develop a strategy to optimally manage this group of patients in recent years.

Resistance to RT (radioresistance) can be intrinsic or acquired (9). Given the heterogeneity of prostate cancer cells, it is likely that certain cells have intrinsic radioresistance, whereas others have the ability to acquire radioresistance over the course of RT. This review discusses the recent advance in our understanding of radiation-induced neuroendocrine differentiation (NED) and the implication on RT efficacy, and proposes possible approaches to addressing radiation-induced NED.

NEUROENDOCRINE DIFFERENTIATION AS A MECHANISM OF THERAPY RESISTANCE

Normal prostate tissue consists of three types of epithelial cells: basal cells, luminal cells, and neuroendocrine (NE) cells. Unlike basal cells and luminal cells, NE cells constitute only <1% of total epithelial cells, and their physiological role remains unclear (10). In prostate adenocarcinoma, the presence of an increased number of neuroendocrine-like (NE-like) cells is observed (10–14). It has been hypothesized that these NE-like cells may arise from luminal-type prostate cancer cells by a NED or transdifferentiation process (15–17). NE-like cells do not proliferate, and lack the expression of androgen receptor (AR) and PSA.

Clinical observations have suggested that NED correlates with disease progression and poor prognosis (14, 16, 18–28). Several mechanisms may account for the impact of NED on prostate cancer progression and therapeutic responses. First, NE-like cells do not proliferate, and thus they function as a dormant phenotype making NE-like cells particularly resistant to therapies. Second, NE-like cells express high levels of survival genes such as survivin and Bcl-2 (29–31), or exhibit alteration in calcium homeostasis

(32), again conferring resistance to treatments. Third, NE-like cells secrete a number of peptide hormones and growth factors to support the growth of surrounding tumor cells in a paracrine manner. Lastly, NED is a reversible process (33, 34). For example, treatment of LNCaP cells with cAMP or cAMP-inducing agents induces NED within a few days (33). Interestingly, removal of cAMP or cAMP-inducing agents results in either retraction or shedding of the neuritic processes within 10 h. Also, within 2 days the expression of neuron specific enolsase (NSE), a biomarker of NE and NE-like cells, returns to basal levels. Similarly, NED induced by androgen depletion (e.g., charcoal-stripped fetal bovine serum-containing medium) can be reversed by culturing cells in normal serum-containing medium. Based on these observations, there are two possible pathways by which NED can contribute to disease progression and therapy failure (**Figure 1**). One is that NE-like cells can survive therapeutic interventions and thus contribute to tumor recurrence if they resume proliferation post treatments. Second, the presence of NE-like cells supports the growth of surrounding tumor cells in a paracrine manner, thus conferring to disease progression.

PRE-EXISTING NED VERSUS THERAPY-INDUCED NED IN PROSTATE CANCER

PRE-EXISTING NED

Although NE-like cells in adenocarcinoma share many characteristics of normal NE cells, they also differ in some aspects. For example, NE-like cells express some luminal cell markers, whereas NE cells express some basal cell markers (15). Accumulating evidence favors the hypothesis that NE-like cells come from a transdifferentiation process of prostate cancer cells, either from hormone-naïve or CRPC (15). There are numerous stimuli and agents, which likely activate distinct signaling pathways to induce NED (15, 35). For example, cAMP signaling may activate the PKA/CREB signaling pathway to induce NED (33, 36–41), whereas IL-6-induced NED appears to be mediated by activation of the PI3K/Etk/Bmx and STAT3 pathways (35, 36, 42–46) (**Figure 1**). Interestingly, while EGF may prevent androgen depletion-induced NED in an MAPK and PI3K/AKT-dependent manner (47), it may also promote NED in LNCaP cells in an ErbB2-dependnt manner if treated with an inhibitor of the PI3K/AKT pathway such as LY294002 (48, 49). Because activation of the cAMP signaling pathway and the PI3K/AKT pathways are often associated with prostate cancer development and progression, it is very likely that a subset of cells may undergo NED during prostate cancer development and progression. Thus, these NE-like cells are already present at the time of initial diagnosis of prostate cancer, and this pre-existing NED confers resistance to subsequent treatments such as RT, androgen-deprivation therapy (ADT), and chemotherapy (14, 16, 18–28).

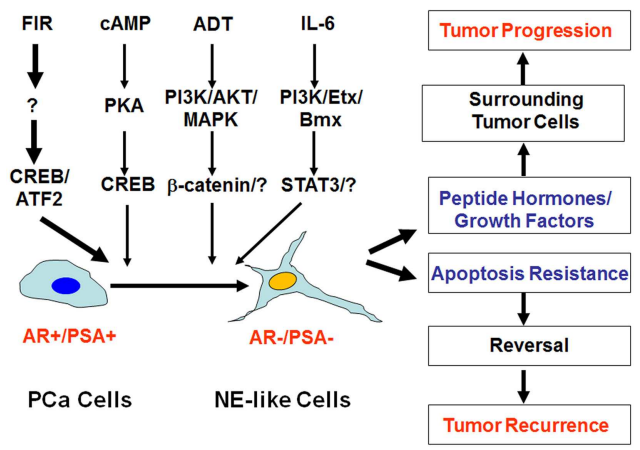

FIGURE 1 | Impact of neuroendocrine differentiation on prostate cancer progression and tumor recurrence. Neuroendocrine differentiation (NED) can be induced by fractionated ionizing radiation (FIR), cAMP, androgen-deprivation therapy (ADT), and IL-6 via distinct signaling pathways. The clinical impact of NED on prostate cancer progression and therapy response can be twofold. On the one hand, NE-like cells can produce peptide hormones and growth factors to promote tumor progression. On the other hand, the dormant and apoptosis-resistant NE-like cells may resume the ability to proliferate due to the reversibility of NED, and contribute to treatment failure and tumor recurrence.

THERAPY-INDUCED NED

Therapy-induced NED refers to acquired NED induced by a therapeutic agent. Such therapeutic agents include ADT (50–52) and docetaxel (23, 53). Recently, it has been shown that enzalutamide and abiraterone (two recently FDA-approved agents for the treatment of CRPC) can also induce NED and that induced NED is correlated with poor survival in CRPC patients (54, 55). Consistent with these clinical observations, induction of NED in prostate cancer cells by androgen depletion is well established *in vitro* (34, 47, 56–59) and in prostate cancer xenografts in mice (59–64). Lin et al. recently reported that a patient-derived xenograft line showed a complete induction of NED following castration (compared to no sign of NED prior to castration) (65). These observations provide convincing evidence that castration does induce NED.

RT CAN ALSO INDUCE NED

While working on the isolation of radiation-resistant sublines after a fractionated RT regimen (2 Gy/day, 5 days/week), we unexpectedly found the display of apparent neurite outgrowth by irradiated cells after a 4-week fractionated ionizing radiation (FIR) (66). Immunoblotting analysis confirmed that these cells express high levels of NE markers chromogranin A (CgA) and NSE, indicating that FIR also induces NED *in vitro*. Furthermore, it was observed that FIR-induced LNCaP xenograft tumors to undergo NED in nude mice, which displayed a four to fivefold increase of serum CgA after 4-week FIR (67). Consistent with this observation, in a pilot clinical study, we measured serum CgA in nine patients who were treated with RT, and found that four out of nine patients showed 1.5- to 2.2-fold increase in serum CgA after 7-week RT (67). Similarly, Lileby et al. also found that a subset of prostate cancer patients treated with RT showed elevated serum CgA levels 3 months after the treatment (21). However, these pilot clinical studies have neither addressed the issue of whether RT-induced CgA elevation correlates with RT failure nor have they established the relationship between the disease status and the extent of serum CgA elevation. Nevertheless, it is clear that NED can be induced by clinical therapeutic agents including RT

(acquired NED), and therapy-induced NED may represent one of the mechanisms leading to treatment failure.

THE RELATIONSHIP BETWEEN NE-LIKE CELLS, CANCER STEM CELLS, SENESCENT CELLS, AND EPITHELIAL-MESENCHYMAL TRANSITION

Based on the expression of marker proteins in NE cells, luminal cells, and basal cells, it was suggested that NE-like cells arise from prostate cancer cells by a process of NED or transdifferentiation (15). However, there is also evidence suggesting that NE-like cells are derived from neural crest cells or stem cells as extensively reviewed by Conteduca et al. (17). Palapattu et al. examined the expression of cancer stem cell marker CD44 in LNCaP, DU-145, and PC-3 cells (68), and revealed that CD44 is only expressed in cells that are positive for NE markers. Consistent with this observation, the correlation between CD44 expression and NE markers (NSE and CgA) was also observed in prostate cancer tissues. Interestingly, 100% of prostatic small cell NE carcinomas, an aggressive variant of prostate cancer that is composed of highly proliferating NE cells, have CD44 expression, whereas its expression was detectable only in a minority of small cell NE carcinoma from other organs. This observation raised an interesting possibility that CD44 expression may be a useful biomarker to distinguish the origin of prostate small cell NE carcinoma from NE carcinoma in other organs. Because CD44 positive cells are capable of generating CD44 negative cells, are highly tumorigenic, and express several "stemness" genes (69), these findings support the hypothesis that CD44 positive NE-like cells are prostate cancer cell stem cells.

Recently, Kyjacova et al. used clinically relevant FIR to irradiate four human prostate cancer cell lines, and observed that there are two populations of survived cells: one is adherent, senescent-like cells, and the other is non-adherent, anoikis-resistant stem cell-like cells (70). However, since the authors did not examine the expression of NE markers, it remains unknown whether one or both populations also express NE markers. We previously isolated several sublines from irradiated LNCaP cells that lost the expression of CgA and NSE (66). All three sublines could not be induced to undergo NED by FIR. Because NED, cancer stem cells, and epithelial–mesenchymal transition (EMT) share similar properties (17), it would be interesting to examine whether these sublines exhibit properties of cancer stem cells, senescent cells, and/or mesenchymal cells. Nonetheless, these observations suggest that FIR treatment may selectively enrich the population of cancer stem cells or induce NED, senescence, and/or EMT. Several mechanisms may account for this. First, NE-like cells, cancer stem cells, and EMT or senescent cells may have the same origin (e.g., stem cells); thus, the type of phenotypic changes may depend on the type of stimuli. Second, NED, cancer stem cells, and EMT or senescence may have a significant overlap of signaling molecules that are required for the development and maintenance of each of these phenotypic changes (17). For example, expression of Snail, a major transcription factor implicated in the induction of EMT, also induces NED in LNCaP cells (71). Third, these phenotypic changes share common inducers, which could lead to induction of NED, stemness, EMT, or senescence. In fact, stress signaling, such as hypoxia, can induce both NED (72) and EMT (73), as well as enrich the cancer stem cell subpopulation (74). Finally, considering cell heterogeneity, the cellular populations may consist of all of these cell types that are induced by distinct stimuli. Future cell lineage analysis and single cell analysis will likely provide insight into the origin of NE-like cells and their relationship with other cell types.

MECHANISM OF RADIATION-INDUCED NED

To study how NED is regulated at the transcriptional level, we examined the subcellular localization of ATF2 and observed increased cytoplasmic localization (66). ATF2 is a member of activator protein 1 (AP-1) family of proteins (75, 76). We discovered that ATF2 is a nucleocytoplasmic shuttling protein that possesses two nuclear import motifs and two nuclear export motifs (77, 78). ATF2 shuttles in LNCaP cells and IR impairs its nuclear import (66). Given that ATF2 belongs to the ATF/CREB family, and CREB is known to both regulate CgA transcription (79) and act downstream of the cAMP signaling (20, 80), we examined the expression and activation of CREB, and found that IR activated CREB as well as increased nuclear localization of phosphorylated CREB at Ser133 (66). These results suggest that CREB is a transcriptional activator of NED while ATF2 is a transcriptional repressor of NED, and that FIR tilts the balance between CREB and ATF2, leading to cell differentiation (**Figure 2**). Indeed, expression of a constitutively activated CREB is sufficient to induce NED, whereas expression of a constitutively nuclear-localized ATF2 (nATF2) can antagonize CREB-induced NED (66). Consistent with the converse roles of CREB and ATF2, nATF2, or a non-phosphorylatable CREB (CREB133A) also inhibits FIR-induced NED. Likewise, we recently established stable cell lines expressing several CREB short hairpin RNAs (shRNAs), and found that CREB knockdown significantly inhibited FIR-induced neurite outgrowth and NSE

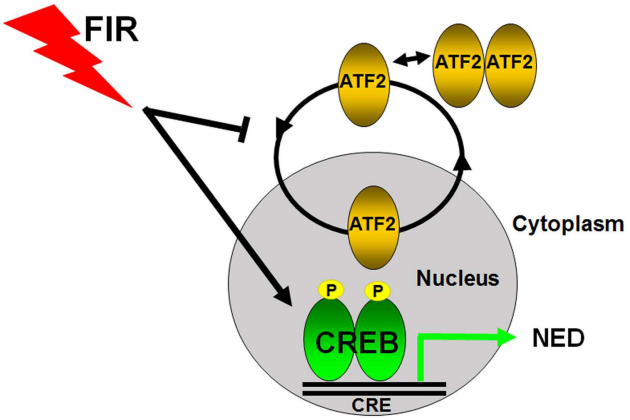

FIGURE 2 | Transcriptional regulation of radiation-induced neuroendocrine differentiation in prostate cancer cells. CREB and ATF2 belong to the same CREB/ATF family transcription factors to regulate gene transcription by binding to the same cAMP response element (CRE). ATF2 constantly shuttles as a monomric form between the nucleus and cytoplasm in prostate cancer cells, and its nuclear regulation is tightly regulated. CREB acts as a transcriptional activator and ATF2 functions as a transcriptional repressor of NED in prostate cancer cells. Fractionated ionizing radiation (FIR) induces NED by activating CREB and impairing the nuclear import of ATF2.

expression (81). However, CgA expression was not inhibited which was surprising given that CREB can activate CgA transcription. Because the CREB family members form different homodimers or heterodimers, the inability of CREB knockdown to inhibit CgA expression may be explained by functional compensation of other dimeric complexes. To overcome this, we established another stable cell line that has inducible expression of ACREB, a dominant negative CREB in which the basic region is replaced by acidic amino acids hence deficient in DNA-binding. This ACREB forms a dimeric complex not only with CREB but also with other CREB family members, exhibiting a potent inhibitory effect on the expression of CREB target genes (82, 83). Indeed, ACREB expression increased radiation-induced cell death by more than 70% in the setting of 40 Gy FIR treatment. Importantly, expression of ACREB both during the first 2 weeks (acquisition of radioresistance) and during the second 2 weeks (acquisition of NED phase) increased FIR-induced cell death (81). This result not only demonstrates the critical role of CREB in FIR-induced NED but also provides evidence that targeting either phase could be an effective approach to developing novel radiosensitizers.

MULTIPLE PHASES OF RADIATION-INDUCED NED

Fractionated ionizing radiation-induced NED differs from androgen depletion- and cAMP-induced NED in that cancer cells must survive from the treatment first. Unlike cAMP- and androgen depletion-induced NED in which almost all LNCaP cells can be induced to differentiate into NE-like cells, we observed that cell growth was largely inhibited during the first week of irradiation, and increased cell death became apparent during the second week of irradiation. However, little cell death was observed starting from the third week onward. Instead, cells began to show neurite outgrowth and cell body became smaller. With continued irradiation, cells showed extended neurite outgrowth (66). Upon 4 weeks of irradiation, almost all survived cells differentiated into NE-like cells and continued irradiation for another 3 weeks did not induce cell death. Similar processes were observed in DU-145 and PC-3 cells, though the extent of NED appears to be less than LNCaP cells (67). These observations suggest that FIR-induced NED constitutes several distinct phases: acquisition of radioresistance during the first 2 weeks, acquisition of NED during the second 2 weeks, maintenance of NED during the last 3 weeks, and reversal to the proliferating state after the completion of the FIR treatment (**Figure 3**).

STRATEGIES TARGETING RADIATION-INDUCED NED

A number of approaches have been attempted to target NE-like cells by either blocking secreted neuropeptide-mediated effects or inhibiting the survival signaling pathways in NE-like cells (17). However, the clinical effect of these therapeutic maneuvers remains unclear. Because NED can be induced by a variety of stimuli and therapeutic agents, the underlying molecular mechanisms of NED need to be thoroughly investigated so that targeted therapies can be developed accordingly. This is particularly important for therapy-induced NED. Further, recurrent tumors derived from therapy-induced NE-like cells may behave differently. For example, RT- and chemotherapy-induced NED involves a clonal selection, and likely reprograming of survival cells. These cells are likely cross-resistant to other treatments (66).

Using radiation-induced NED as a model, we hypothesize here that two complementary directions could be pursued to develop novel therapeutics. One is to identify targets and pathways that are specific for the acquisition of radioresistance and NED, and the other is to identify molecules that are critical for the maintenance of NE-like phenotype. In addition, developments of agents

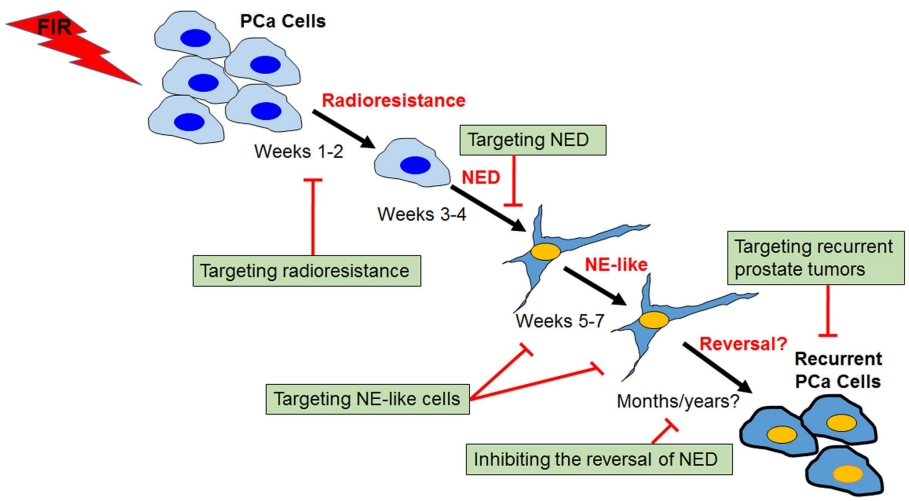

FIGURE 3 | Process and targeting strategies of radiation-induced neuroendocrine differentiation in prostate cancer cells. Shown is a schematic view of several distinct phases of fractionated ionizing radiation (FIR)-induced NED in prostate cancer cells (PCa). The critical role of CREB in the acquisition of radioresistance and NED phases has been demonstrated, and identification of upstream regulators of CREB may lead to development of novel radiosensitizers. Targeting NE-like cells and inhibiting the reversal of the "dormant" NE-like cells to a proliferating state could also be clinically useful. Further, profiling radioresistant recurrent prostate cancer cells may allow identification of molecules contributing to cross-resistance of recurrent prostate cancer after radiotherapy failure, and ultimately may lead to the development of novel therapeutic agents for the treatment of recurrent prostate tumors.

that inhibit the reversal of NE-like cells or target recurrent tumors after RT failure should also be considered.

TARGETING ACQUISITION OF RADIORESISTANCE AND DIFFERENTIATION PHASES

Because NED can be induced by a variety of stimuli via activation of distinct mechanisms, targeting specific signaling pathways downstream of a particular inducer is a reasonable strategy. Application of such targeting agents (applied as either a single agent or a combination of multiple agents) would therefore inhibit therapy-induced NED. In the case of RT-induced NED, we have demonstrated that the CREB signaling is critical for FIR-induced NED (66, 67). To determine whether targeting RT-induced NED can be explored to develop a novel radiosensitizer, we established doxycycline-inducible expression system to diminish CREB activity by expressing either ACREB, a dominant negative mutant of CREB, or shRNAs to knockdown CREB. The availability of these two inducible CREB targeting approaches allowed us to specifically test whether targeting CREB during the first 2 weeks or during the second 2 weeks can sensitize prostate cancer cells to radiation. Our results showed that targeting CREB during either phase can increase FIR-induced cell death (81). This finding not only confirms that CREB is critical for FIR-induced NED but also suggests that targeting FIR-induced NED can sensitize prostate cancer cells to radiation. Since several CREB targeting agents are being developed (84), it would be interesting to test whether these agents are effective in inhibiting FIR-induced NED. Furthermore, identification of upstream regulators, e.g., protein kinases, could provide an important approach to targeting FIR-induced NED. In conclusion, this type of targeting agents can be developed as radiosensitizers by targeting either the acquisition of radioresistance, NED phase, or both phases.

TARGETING NE-LIKE CELLS

Because NE-like cells do not proliferate and rather stay as "dormant" cells, cytotoxic chemotherapeutic agents may not be effective. It is therefore necessary to understand how these "dormant" cells survive and maintain their phenotype. It is possible that an autocrine pathway confers cell survival and would be a potential target for therapeutics. Alternatively, we may target the survival pathway. For example, NE-like cells often overexpress survivin (29), and several survivin-targeting agents have been developed (85). It would be interesting to determine if targeting survivin can induce apoptosis of therapy-induced NE-like cells.

INHIBITING THE REVERSAL OF NE-LIKE CELLS

One of the potential impact of NED on tumor recurrence is its reversibility. Like cAMP- and androgen depletion-induced NED (33, 34, 58), FIR-induced NED may also be reversible (66). The molecular mechanisms underlying this process remain unclear. However, inhibiting the reversal of NE-like cells to a proliferating state may be clinically useful if the reversibility of NE-like cells does occur in prostate cancer patients.

TARGETING RECURRENT PROSTATE CANCER CELLS

Treatment of recurrent prostate cancer remains a major challenge. A therapy for recurrent tumor is variable, and depends on the type of primary treatment. For example, a treatment strategy for recurrent prostate cancer after RT failure is different from that for recurrent prostate cancer after surgery. This is because recurrent prostate cancer after RT has undergone genetic and epigenetic changes under the selective pressures, and may be cross-resistant to other treatments. Consistent with this notion, isolated radioresistant sublines after 40 Gy of FIR are indeed cross-resistant to androgen depletion and docetaxel (66). Given that 30–50% of high-risk and 10% of low-risk prostate cancer recur after RT, it is urgently needed to develop agents that can specifically target recurrent prostate cancer after RT failure. Because the recurrent tumor is composed of heterogeneous cells, including NE-like cells or cancer stem cells as discussed above, comparative analysis of genetic and epigenetic changes as well as signaling pathways between multiple radioresistant sublines and parental cells may lead to identification of molecular alterations that are common to all recurrent cells. If identified, molecular alterations could be validated with recurrent prostate cancer specimens, and developing novel therapeutics targeting specifically for RT-failed recurrent prostate cancer may become possible.

FUTURE PERSPECTIVES

ANIMAL MODELS TO STUDY THE IMPACT OF NED IN PROSTATE CANCER PROGRESSION AND THERAPEUTIC RESPONSE

The impact of NED on prostate cancer progression has been well demonstrated *in vivo*. It was shown that the implantation of NE mouse prostate allograft (NE-10) in nude mice bearing LNCaP xenograft tumors on the opposite flank can support the growth of LNCaP xenograft tumors under castration condition (86). This study provides compelling evidence that factors secreted by NE tumors are sufficient to support the growth of prostate tumors under castration condition (86). Consistent with this, Deeble et al. elegantly demonstrated again in castrated condition that coinjection of the constitutively activated protein kinase A subunit-induced NE-like cells and LNCaP cells into nude mice enhanced tumor growth (38). These studies corroborated *in vitro* findings that conditioned medium from NE-like culture can stimulate the growth of prostate cancer cells (38, 87), and that secreted mitogenic neuropeptides such as neurotensin are critical for the stimulation of tumor cell growth (33, 36, 87). Interestingly, Valerie et al. also showed that treating prostate cancer cells expressing high levels of neurotensin receptor 1 (NTR1) with a selective NTR1 antagonist SR48692 sensitizes prostate cancer cells to ionizing radiation. Thus, secreted neurotensin from NE-like cells not only promotes prostate cancer cell growth but also confers the surrounding tumor cells radioresistance. Although these studies provide evidence that secreted neuropeptides and growth factors from NE-like cells *in vivo* can promote prostate cancer progression and alter therapeutic responses, these findings are limited to established cell lines in immunocompromised mice and thus further research must be done with a better model system.

While many genetically engineered mouse (GEM) models have been established to study the development, progression, and therapeutic responses of prostate cancer (88), a GEM model that allows for the elucidation of the impact of NED on prostate cancer progression and therapeutic response is unavailable. By transgenically overexpressing SV40 large T antigen, a TRAMP mouse model was

established, which has a high incidence of NE tumor arising from prostate with a high potential to metastasize to lung, liver, and other tissues (89). The TRAMP mouse model is more representative of human NE carcinoma, a rare type of prostate cancer present at initial clinical presentation or in some ADT-treated setting (88). Recently, Qi et al. found that knockout of Siah2, a ubiquitin ligase, completely suppresses the development of NE tumors in the background of TRAMP (90), demonstrating a critical role of this E3 ligase in the development of NE tumors. Molecular analysis further revealed that HIF-1α, which is stabilized by Siah2, mediates the effect of Siah2 to selectively regulate, in combination with FoxA2, the expression of HIF target genes that are required for or involved in the development of NE tumor. Although these studies provide genetic evidence that Siah2, HIF-1α, and FoxA2 are required for the regulation of NE tumor development at the transcription level, the TRAMP mouse model does not permit the analysis of the impact of pre-existing and therapy-induced focal NED on disease progression and therapeutic response. Given that castration-induced NED also occurs in other GEM models (91, 92), it would be interesting to test if FIR also induces NED in these GEM models. Further, innovative approaches (e.g., inducible NED mouse models, chemical probes) that allow manipulation of NE-like cells or NED in these GEM models will likely facilitate the study of NED impact on prostate cancer progression and radiation response. As castration-induced NED has also been reported in patient-derived xenograft model system (65), infecting the cells with lentiviruses (that can inducibly destroy NE-like cells during the course of FIR treatment) will similarly permit the study of acquired NED in radio-responsiveness.

CLINICAL DIAGNOSIS OF NED IN PROSTATE CANCER PATIENTS

Traditionally, the proteins such as CgA, NSE, synaptophin, and others that are expressed by NE-like and NE cells are used as biomarkers to identify NE-like or NE cells in tissue specimens using immunohistochemistry. However, analysis is often confounded by various factors including a sampling issue, leading to conflicting outcomes. Thus, it is generally felt that immunohistochemical analysis may not accurately represent the status of NED in a given patient. To overcome this, serum biomarkers have been used and their correlation to NED in tissues have been examined. It was found that CgA is the best biomarker to reflect NED in tissue (93). To date, serum CgA has been used to monitor ADT-induced NED and chemotherapy-induced NED (23, 24, 27, 53–55, 94, 95). We and others have also observed serum CgA elevation in some patients who were treated with RT (21, 67). Because prostate cancer cells express a basal level of CgA, and activation of transcription factors (e.g., CREB) may also lead to increased synthesis of CgA, measurement of individual biomarkers may not accurately reflect the status of NED in tissues. In addition, obtaining a biopsy for the examination of NED in cancer tissues in post-RT setting is very challenging. Thus, it is very desirable to develop new methods that can reliably diagnose NED in cancer tissues. One approach is to test whether circulating tumor cells can be used to monitor NED in patients in addition to serum CgA measurement. Alternatively, measurement of multiple biomarkers may be necessary for a more accurate diagnosis. One example is the ratio of CgA/PSA. Measurement of serum CgA in irradiated xenograft tumors revealed that the ratio of serum CgA/PSA might provide a better prediction of NED (67). Given that NE-like cells are PSA-low or negative and can secrete CgA, future research should focus on their relationship and the correlation with clinical outcomes.

POTENTIAL IMPACT OF CURRENT TREATMENT MODALITIES ON RADIOTHERAPY-INDUCED NED

Evaluation of current treatment modalities for locally advanced diseases

Locally advanced, high-risk, prostate cancer currently poses therapeutic challenges. Currently, the standard management for this group of patients is a combined treatment of RT plus ADT. The rationale for combining RT with ADT was based on the fact that both treatments can kill cancer cells or suppress cancer cell growth, and that the combination may lead to a synergistic effect. Indeed, several phase III clinical studies have demonstrated that RT plus ADT provides a survival benefit, in comparison with either RT or ADT alone (4, 96–99). The rationale for adding ADT in the RT setting is that ADT can eliminate androgen-dependent clones, potentiate the tumoricidal effect of RT, and may eradicate micrometastatic disease (96). However, whether ADT can radiosensitize prostate cancer cells is unknown. In fact, *in vitro* studies using LNCaP cells suggest that androgen depletion did not radiosensitze LNCaP cells in clonogenic assays, though apparent additive effect was observed (100). Given that ADT induces NED in a subpopulation of cancer cells (50–52), it would be necessary to evaluate the impact of this combined therapeutic approach on therapy-induced NED, in comparison to a monotherapy setting (ADT or RT alone). Ideally, developing novel therapeutic agents that not only sensitize prostate cancer cells to RT but also inhibit therapy-induced NED would be ideal and likely initiate a paradigm shift for future management of prostate cancer.

Impact of new treatment modalities on RT-induced NED

Radiotherapy is one of the main curative modalities for localized prostate cancer. Advances have been made to improve the efficacy of RT in recent years. These include a dose-escalation strategy, a hypofractionation regimen, an incorporation of chemotherapy, and a new RT modality such as high-dose-rate brachytherapy and proton therapy (101–108). Although biological, physical, and clinical rationales clearly support the use of these treatment modalities, their impact on radiation-induced NED remains unstudied. It is worth mentioning that all nine patients enrolled in our pilot clinical study were treated with proton therapy (67). As such, it could be critical to compare the effect of various other RT protocols or modalities on radiation-induced NED. Because FIR-induced NED is completed by a 4-week of irradiation, a dose-escalation strategy over a protracted course likely has a minimal effect on radiation-induced NED. However, other treatment strategies such as an ultra-hypofractionation regimen (e.g., five treatments over 1–2 weeks) or high-dose-rate brachytherapy (given over 1–2 weeks) may have less extent of radiation-induced NED. Also, proton therapy may have less degree of radiation-induced NED, as it has a higher relative biological effectiveness in comparison to a conventional photon beam. The decrease in radiation-induced NED may, in turn, translate to a clinical benefit with improved treatment outcomes. On a translational research perspective, it would

be worthwhile to determine whether the observed clinical benefit correlates with the extent of radiation-induced NED. If so, this would provide a biological rationale for exploring different RT regimens or modalities aiming to minimize radiation-induced NED and may also allow for reduction or possible elimination of the use of adjuvant ADT in RT setting.

CONCLUSION

Although NED has been a well-recognized phenotypic change in prostate cancer, its impact on prostate cancer progression and therapeutic responses has only recently gained significant attention. Several studies have provided compelling evidence that pre-existing NED confers resistance to treatments such as RT. However, the impact of therapy-induced NED on disease progression and treatment failures has not been rigorously studied. Using FIR-induced NED as a model system, we have provided evidence that targeting FIR-induced NED is an effective radiosensitizing approach. Future research should be directed at understanding the molecular mechanisms by which FIR induces NED and confers acquired radioresistance as well as tumor recurrence. With the use of appropriate animal models, implementation of new technologies as well as methodologies to diagnose RT-induced NED and better understanding of the biological effect of novel treatment modalities, we hope that a better RT strategy will be developed and implemented in clinical practice in the future.

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Education / Degrees Awarded:

9/1979-7/1984: Bachelor in Medical Science (Equivalent to ***M.D.***)
Faculty of Medicine, Bengbu Medical College, Bengbu, China
9/1984-7/1987: ***M.S.*** (Cancer Immunology)
Department of Microbiology and Immunology, College of Medicine, Tongji Medical University, Wuhan, China
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Department of Physiology II, Kobe University School of Medicine, Japan

Research/Working Experience:

9/1984-7/1987: ***Graduate Student (M.S.)*** in the Department of Microbiology & Immunology, Tongji Medical University, Wuhan, China.
Study of anti-tumor mechanisms of a new Chinese herb in cell culture and animal models.
7/1987-9/1991*:* ***Lecturer*** in the Department of Epidemiology, School of Public Health, Tongji Medical University, Wuhan, China.
(1). Mutagenicity of trichloromethane in drinking water
(2). Epidemiological investigation of drinking water and cancer incidence in Wuhan, China.
9/1991-3/1994: ***Visiting Research Associate*** in the Department of Molecular Oncology, Kyoto University School of Medicine, Kyoto, Japan.
(1). Spontaneous and induced acquisition of tumorigenicity in nude mice by lymphoblastoid cell line derived from patients with xeroderma pigmentosum group A.
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4/1994-3/1997: ***Graduate Student (Ph.D.)*** in the Department of Physiology II, Kobe University School of Medicine, Kobe, Japan
(1). Identification of cysteine-rich domain in Raf-1 as a novel Ras binding domain for activation by Ha-Ras and Rap1A.

(2). Activation mechanisms of Ras effectors (Raf-1, B-Raf, adenylyl cyclase).

4/1997-8/2000: ***Assistant Professor*** in the Department of Physiology II, Kobe University School of Medicine, Kobe, Japan.

(1). Differential regulation of Raf kinase activity by Ha-Ras and Rap1A.

(2). Identification and characterization of novel Ras effectors, (RalGDS, AF-6, PLC-ε) and regulators (RA-GEF-1, RA-GEF-2).

(3). Activation mechanisms of Ras effectors.

9/2000-6/2003: ***Research Investigator/Specialist*** in the Department of Biological Chemistry and Howard Hughes Medical Institute, University of Michigan School of Medicine.

(1). Development of bimolecular fluorescence complementation (BiFC) and multicolor BiFC assays for visualization of protein-protein interactions in living cells.

(2). Functional analysis of cross-family transcription factor interactions among bZIP, Rel, Smad and Myc/Max families.

7/2003-6/2009: ***Assistant Professor*** in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University College of Pharmacy.

(1) Development and improvement of BiFC-based technologies
(2) BiFC analysis of AP-1 dimers in living cells and *C. elegans*
(3) AP-1 in prostate cancer development and therapeutic responses

7/2009- 7/2015: ***Associate Professor*** (tenured) in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University College of Pharmacy.

(1) Development and improvement of BiFC-based technologies
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(3) Mechanisms and targeting of radiation-induced neuroendocrine differentiation in prostate cancer
(4) Protein arginine methyltransferase 5 (PRMT5) in prostate cancer development, progression and therapeutic response

8/2015- present: ***Professor*** (tenured) in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University College of Pharmacy.

(1) Mechanisms and targeting of radiation-induced neuroendocrine differentiation (NED) in prostate cancer
(2) Role and targeting of protein arginine methyltransferase 5 (PRMT5) in castration resistant prostate cancer (CRPC) and neuroendocrine prostate cancer (NEPC)
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(4) Development of BiFC-based cDNA library screens for interacting proteins

08/2013-present: Program Co-Leader of the Cell Identity and Signaling (CIS) program of the Purdue University Center for Cancer Research (PCCR)

08/2013-present: Executive Committee Member of PCCR

08/2010-present: Co-Leader of the Prostate Cancer Discovery Group of PCCR
2011-present: Director of Pharmacy Live Cell Imaging Facility (PLCIF)
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Current Professional Memberships

2001- Present	American Association for Cancer Research
2009- Present	Society for Basic Urological Research
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Awards:

09/91-09/92:	Fellowship of JSPS Source: **J**apan **S**ociety for the **P**romotion of **S**cience (JSPS)
09/92-09/93:	Kyoto University Alumni Fellowship Source: Kyoto University
04/94-03/97	Senshukai Scholarship (Ph.D. student) Source: Kobe Senshukai Scholarship Foundation
04/98-03/99	President Young Investigator Award Source: Kobe University
04/98-03/99	Young Investigator Award Source: JSPS
04/99-03/01	Young Investigator Award Source: Hyogo Prefecture Science and Technology Association
07/03-08/06	Walther Assistant Professor
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Professional Services:

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2004	Reviewer of MAES (The Maryland Agricultural Experiment Station at the University of Maryland)
2005	Reviewer for NSF Advisory Panel for Molecular and Cell Biology
2006-2008	American Heart Association (MCB Panel)
2007-2011	Qatar National Research Fund (QNRF)
2008-present	Pennsylvania Department of Health (PADOH)
2008	UK Cancer Research
2008	UK Diabetes
2009	Welcome Trust

2010-2014	Department of Defense, Prostate Cancer Research Program (Immunology, Endocrine, Experimental Therapeutics panels)
2015/2016	Florida Department of Health
2015	NIH, RTB study section (IAR)
2016	NCI (DP5)

Reviewer for Professional Journals

Combinatory Chemistry and HTS, Zebrafish, Journal of Biological Chemistry, Molecular and Cellular Biology, Nature Biotechnology Nature Methods, Molecular Cell, Molecular Biology of the Cell, PNAS, BMC Biotechnology, BMC Biology, Biotechniques, Biochemistry, ACS Chemical Biology, Chemistry & Biology, Journal of Innovative Optical Health Sciences, TIBS, TIBT, Current Cancer Drug Targets, Journal of Cell Science, PLoS One, Ontarget, Oncogene, Redox Biology, Cancer Letters

Editorial Board Member:

2007- Perspective in Medicinal Chemistry
2011- American Journal of Cancer Research
2013- Journal of Biological Methods (Founding Editorial Member)
2014- Frontier in Surgical Oncology (review editor)
2015- Journal of Drug Research and Development

Organizer/Program Committee Member/Session Chair of Conferences, Symposiums, and Workshops

- Organizer of Tristate Worm Meeting at Purdue (2006)
- Session Chair of Optical Molecular Imaging of the 2008 PIBM
- Session Chair of Imaging Technology Symposium of the 2008 4th Modern Drug Discovery and Development Summit
- Program Member of the 2009 PIBM Program Committee
- Organizer of 2010 Bimolecular Fluorescence Complementation Workshop (Purdue University)
- Member of the Scientific Program Committee and Moderator of Breakout Panel Discussion of the 2013 Drug Discovery Chemistry-Sixth Annual Protein-Protein Interactions, San Diego
- Organizer, Program Committee Member and Session Chair of the 2013 Hefei Prostate Cancer Translational Medicine and Personalized Medicine Symposium
- Session Co-chair of the 2016 Spring SBUR Symposium

Member of Big Ten Cancer Research Consortium (BTRC) GU Clinical Trial Working Group (2013-present)

Consultation on BiFC technology

Since 2003, we have been providing BiFC plasmids, letters of support and consultations to many BiFC users worldwide. The lab provided BiFC plasmids to more than 200 labs prior to 2007. To facilitate the request process, we deposited 11 BiFC plasmids to Addgene in 2007, and more than 2000 requests have been completed via Addgene.

Invited Seminars/Presentations

07/04/17	Place: China Jiliang University School of Pharmacy Title: Title: Title: Bimolecular fluorescence complementation (BiFC): From basic research to drug discovery
06/16/17	Place: Hong Kong University School of Chinese Medicine Title: Bimolecular fluorescence complementation (BiFC): From basic research to drug discovery
06/12/17	Place: Jinan University School of Medicine Title: Protein arginine methyltransferase 5 (PRMT5): An emerging oncogene and therapeutic target in prostate cancer
05/15/2017	Place: Northwesten University School of Medicine, Department of Pathology Title: Neuroendocrine differentiation of prostate cancer: An emerging mechanism of therapy resistance
10/11/2016	Place: Chromatin and Epigenetics Symposium (Purdue) Title: PRMT5 is a master epigenetic activator of DNA damage response and a therapeutic target for prostate cancer radiosensitization (presented by Jake Owens)
05/10/16	Place: 2016 American Urological Association (AUA) meeting Title: Protein arginine methyltransferase 5 (PRMT5) is a novel epigenetic regulator of androgen receptor in prostate cancer
01/07/16:	Place: Jinan University the first affiliated hospital Title: How to conduct scientific research
12/27/15:	Place: Northwest University of Agriculture and Forestry Title: Bimolecular fluorescence complementation (BiFC): Current status and future perspectives
01/05/15:	Place: Tongling First People's Hospital Title: Advances in prostate cancer diagnosis and treatment- A comparative analysis between China and America
12/29/14	Place: Jinan University the first affiliated hospital Title: Targeting PRMT5 for prostate cancer radiosensitization
05/18/14	Place: Mayo Clinic, Departments of Radiation Oncology Title: Mechanism and targeting of radiotherapy-induced neuroendocrine differentiation for prostate cancer treatment
03/25/14	Place: Tongling 4th Hospital, Wannan Medical College Title: Advances in prostate cancer diagnosis and treatment
02/27/14	Place: UCLA, Departments of Pathology and Laboratory Medicine

	Title: Targeting neuroendocrine differentiation as a novel radiosensitization approach for prostate cancer treatment
10/9//13	Place: Cancer Hospital, Hefei Institutes of Physical Science Chinese Academy of Sciences Title: Development of radiosensitizers: An urgent need for prostate cancer radiotherapy
05/24/13	Place: Hefei Chinese Academy of Sciences Cancer Hospital Title: Impact of neuroendocrine differentiation in prostate cancer radiotherapy
05/20/13	Place: Huazhong University of Science and Technology Union Hospital Cancer Institute Title: Radiation-induced neuroendocrine differentiation in prostate cancer: From bench to bedside
05/17/13	Place: Jinan University School of Medicine Title: Neuroendocrine differentiation (NED) in prostate cancer cells: From basic science to clinical practice
05/14/13	Place: Northwestern Agriculture and Forestry University (NWAFU): 2013 Purdue-NWAFU Center Symposium Title: Bimolecular fluorescence complementation (BiFC): Current Status and Future Perspectives
04/17/13	Place: 2013 Drug Discovery Chemistry in San Diego: Sixth Annual Protein-Protein Interactions (Targeting PPI for Therapeutic Interventions) Title: Bimolecular fluorescence complementation (BiFC) as a novel imaging-based screening for inhibitors of protein-protein interactions.
02/05/13	Place: Tongji Hospital, Huazhong University of Science and Technology Title: Neuroendocrine differentiation (NED): A therapeutic challenge in prostate cancer management
10/25/12	Place: Wright State University Department of Biochemistry and Molecular Biology Title: Bimolecular fluorescence complementation (BiFC): An imaging tool for visualization of molecular events
06/06/12	Place: Jiangshu University School of Medical Technology and Laboratory Medicine Title 1: Mechanisms and targeting of radiation-induced neuroendocrine differentiation Title 2: Bimolecular fluorescence complementation (BiFC): Past, Present and Future
06/4/12	Place: Chinese Academy of Sciences (Hefei) Title: Bimolecular fluorescence complementation (BiFC): Past, Present and Future
05/31/12	Place: Tongling Traditional Chinese Medicine Hospital Title: Recent advances in prostate cancer diagnosis and treatment

05/18/12	Place: Shanghai Center for Plant Stress Biology of Chinese Academy of Sciences Title: Bimolecular fluorescence complementation (BiFC): Past, Present and Future
04/25/12	Place: University of Western Ontario Title: Radiotherapy-induced neuroendocrine differentiation: Implications in prostate cancer progression and treatment
03/13/12	Place: Mayo Clinic Department of Urology Title: Mechanisms and targeting of therapy-induced neuroendocrine differentiation for prostate cancer treatment
07/11/11	Place: Jinan University Medical School Title: Bimolecular fluorescence complementation: An emerging technology for biological research
07/10/11	Place: Sun-Yat-sun University Medical School Title: Mechanisms and targeting of therapy-resistant prostate cancer
02//09/11	Place: Tulane University Medical School Title: Mechanisms and targeting of therapy-resistant prostate cancer
01/17/11	Place: Penn State University College of Medicine Title: Bimolecular fluorescence complementation (BiFC): Current Challenges and Future Developments
12/07/10	Place: Purdue University BiFC Workshop Title: Bimolecular fluorescence complementation: principle, experimental design and data analysis
11/18/10	Place: UT Austin College of Pharmacy Title: Bimolecular fluorescence complementation (BiFC) analysis of AP-1 dimierzation in living cells and *C. elegans*
09/28/10	Place: Nanjing University Medical School Title: Multicolor bimolecular fluorescence complementation (BiFC): A novel high throughput screening method for protein-protein interactions
09/25/10	Place: Wannan Medical College Title: Mechanisms and targeting of therapy-resistant prostate cancer
09/16/10	Place: Wuhan Institute of Virology Title: Bimolecular fluorescence complementation (BiFC): Current Status and Future Perspectives
09/13/10	Place: Beijing University Cancer Hospital Title: Mechanisms and targeting of therapy resistant prostate cancer
09/08/10	Place: Purdue University BIG Symposium Title: Fluorescence complementation: An emerging tool for visualization of molecular events in living cells and animals
10/16/09	Place: Southern China Agriculture University

	Title:	Principle and applications of bimolecular fluorescence complementation (BiFC)
10/19/09	Place:	Sun Yat-sen University Zhongshan Medical School
	Title:	Principle and applications of bimolecular fluorescence complementation (BiFC)
10/26/09	Place:	Bengbu Medical College
	Title:	Principle and applications of bimolecular fluorescence complementation (BiFC)
10/28/09	Place:	Nanjing University Medical School
	Title:	Seeing is believing: visualization of protein-protein interactions using bimolecular fluorescence complementation (BiFC),
05/07/09	Place:	University of Chicago Graduate Program of Physiology
	Title:	Bimolecular fluorescence complementation (BiFC) analysis in living cells and living animals,
02/02/09	Place:	Indiana University Medical School, Department of Biochemistry
	Title:	Ionizing radiation-induced neuroendocrine differentiation: implication in prostate cancer therapy
12/08/08	Place:	University of Virginia Cancer Center
	Title:	Ionizing radiation-induced neuroendocrine differentiation: implication in prostate cancer therapy
11/25/08	Place:	7th International Conference on Photonics and Imaging in Biology and Medicine (Wuhan, China), Nov 24-27, 2008
	Title:	Fluorescence complementation: an emerging technology in biomedical research (presentation and panel discussion)
10/15/08	Place:	4th Modern Drug Discovery & Development Summit (San Diego, 10/15/08-10/17/08)
	Title:	Multicolor bimolecular fluorescence complementation in drug discovery
11/29/07	Place:	UMDNJ-SOM Stratford
	Title:	Bimolecular fluorescence complementation (BiFC) analysis of AP-1 dimerization in living cells and living animals
11/28/07	Place:	The Children's Hospital of Philadelphia and the University of Pennsylvania
	Title:	Molecular regulation and targeting of ATF2 nucleocytoplasmic shuttling
11/13/07	Place: Department of Biochemistry, Purdue University	
	Title: AP-1 biology, pathology, and technology	
10/30/07	Place:	Fluorescent proteins and Biosensors Symposium at HHMI Janelia Farm
	Title:	BiFC-FRET, a novel assay for visualization of ternary complexes in living cells
08/07/07	Place:	International Microscopy & Microanalysis 2007 at Ft. Lauderdale
	Title:	Bimolecular fluorescence complementation (BiFC) and

beyond

02/09/07 Place: Montana State University Department of Microbiology
Title: Functional analysis of AP-1 dimerization by bimolecular fluorescence complementation

11/01/06 Place: Vanderbilt University Institute of Chemical Biology
Title: Visualization of AP-1 protein interactions in living cells and in living animals using an improved BiFC system

10/04/06 Place: University of Illinois at Chicago School of Medicine
Title: Bimolecular fluorescence complementation: principle and applications

07/17/06 Place: Huazhong University of Science and Technology Tongji Medical College
Title: Bimolecular fluorescence complementation: principle and applications

03/14/06 Place: University of Toronto Western Research Institute
Title: Visualization of AP-1 protein interactions in living cells and in living animals using an improved BiFC system

09/30/05 Place: Eli Lilly, Indianapolis
Title: Identification of new fluorescent protein fragments for BiFC analysis under physiological conditions

03/10/05 Place: Purdue University, School of Health Science, Purdue University
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions

09/02/04 Place: Illinois State University, Department of Biology
Title: Role of *C. elegans* Fos and Jun homologs in development.

08/13/04 Place: Cold Spring Harbor (Cold Spring Harbor Image Course)
Title: Seeing is believing: visualization of transcription factor interactions in living cells and in living animals using a novel using bimolecular fluorescence complementation (BiFC) approach

05/07/04 Place: Purdue University, Department of Chemistry
Title: Seeing is believing: visualization of transcription factor interactions in living cells and in living animals

01/14/04 Place: Purdue University, Department of Biological Science
Title: Seeing is believing: visualization of transcription factor interactions in living cells and in living animals

12/04/03 Place: Indiana University at Bloomington, Department of Biology
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions

11/07/03 Place: Purdue Cancer Center (Purdue Cancer Center Director's Advisory council)
Title: Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions in cancer research

09/04/03 Place: Purdue Cancer Center (Annual Scientific Retreat)

	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interactions
03/11/03	Place:	Cincinnati Children's Hospital, Division of Experimental Hematology
	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells
03/04/03	Place:	Harvard Medical School, MGH, Laboratories of Photomedicine
	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells
02/24/03	Place:	Medical University of South Carolina, School of Pharmacy Department of Pharmaceutical Science
	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells
02/19/03	Place:	University of Texas M.D. Anderson Cancer Center, Department of Molecular Therapeutics
	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells
02/06/03	Place:	Ohio State University, School of Medicine Department of Physiology and Cell biology
	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells
12/28/02	Place:	Purdue University Cancer Center
	Title:	Bimolecular fluorescence complementation (BiFC), a novel approach to study protein-protein interaction in living cells
07/20/00	Place:	Bengbu Medical College, Bengbu, China
	Title:	Recent progress in the activation mechanisms of Raf by Ras
07/15/00	Place:	Tongji Medical University, Wuhan, China
	Title:	Cloning and functional characterization of a novel type phospholipase C (PLC-ε)

Development of Intellectual Property

- A novel fluorescent protein for protein-protein interaction studies, 65557.P1.US Patent filed on July 16, 2010
- Methods for identifying protein-protein interactions, 66261-01-2013 US Patent filed on June 13, 2013
- Methods for identifying protein-protein interactions, 66261-02-2014 US Patent filed on June 14, 2014
- Bimolecular fluorescence complementation (BiFC)-based screen for discovery of PRMT5 inhibitors. Provisional Patent Application No 62/121,627 filed on February 27, 2015

Publications

a. Peer-reviewed Research Articles

Zeng, L., Wang, W.H., Arrington, J., Shao, G., Geahlen, R.L., Hu, C.D. and Tao, W.A. Identification of upstream kinases by fluorescence complementation mass spectrometry. *ACS Central Sci*, http://pubs.acs.org/doi/pdf/10.1021/acscentsci.7b00261 (2017)

Deng, X., Shao, G., Zhang, H.T., Li, C., Zhang, D., Cheng, L., Elzey, B.D., Pili, R., Ratliff, T.L., Huang, J., Hu, C.D. Protein arginine methyltransferase 5 functions as an epigenetic activator of the androgen receptor to promote prostate cancer cell growth. *Oncogene*, 36:1223-1231 (2017)

Vickman, R.E., Christ, S.A., Kerian, K., Eberlin, L., Coos, R.G., Burcham, G.N., Buhman, K.K., Hu, C.D., Mesecar, A.D., Cheng, L., Ratliff, T.L. Cholesterol sulfonation enzyme, SULT2B1b, modulates AR and cell growth proerties in prostate cancer. *Mol Cancer Res*, 14:776-786 (2016)

Zhang, H., Zeng, L., Tao, A.W., Zha, Z., and Hu, C.D*. The E3 ubiquitin ligase CHIP mediates ubiquitination and protesomal degradation of PRMT5. *Biochem Biophys Acta*, 1863:336-346 (2016)

Xu, D., Zhan, Y., Qi, Y., Cao, B., Bai, S., Xu, W., Gambhir, S.S., Lee, P., Sartor, O., Flemington, E.K., Zhang, H., Hu, C.D., and Dong, Y. Androgen receptor splice variants dimerize to transactivate target genes. *Cancer Res*, 75:3663-3671 (2015)

Suarez, C.D., Deng, X., and Hu, C.D.* Targeting CREB inhibits radiation-induced neuroendocrine differentiation and increases radiation-induced cell death in prostate cancer cells. *Am J Cancer Res*, 4:850-861 (2014)

Zhang, H., Zha, Z. and Hu, C.D*. Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells. *Biochem Biophys Acta*, 1839:1330-1340 (2014)

Hsu, C. and Hu, C.D.* Transcriptional activity of c-Jun is critical for the suppression of AR function. *Mol. Cell. Endocrinol*. 372:12-22 (2013)

Young MM, Takahashi Y, Khan O, Park S, Hori T, Yun J, Sharma AK, Amin S, Hu CD, Zhang J, Kester M, Wang HG. Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation and apoptosis. *J. Biol. Chem*. 287:12455-12688 (2012)

Hsu, C. and Hu, C.D.* Critical role of an N-terminal end nuclear export signal in regulation of ATF2 subcellular localization and transcriptional activity. *J. Biol. Chem*. 287:8621-8632 (2012)

Deng, X., Elzey, B.D, Poulson, J.M., Morrison, W.B., Ko, S.C., Hahn, N.M., Ratliff, T.L., and Hu, C.D.* Ionizing radiation induces neuroendocrine differentiation in vitro, in vivo and in human prostate cancer patients. *Am. J. Cancer. Res.* 1:834:844 (2011)

Xing, J., Wang, S., Lin, F., Pan, W., Hu, C.D., and Zheng, C. A comprehensive characterization of interaction complexes of Herpes Simplex Virus type 1 ICP22, UL3, UL4 and UL20.5. *J. Virol.* 85:1881-1886 (2011)

Kodama, Y. and Hu, C.D.* An improved bimolecular fluorescence complementation assay with high signal-to-noise ratio. *Biotechniques,* 49:793-805 (2010)

Le, T.T, Duren, H.M., Slipchenko, M.N., Hu, C.D.* and Cheng, J.X. Label-free quantitative analysis of lipid metabolism in living *Caenorhabditis elegans. J. Lipid Res.* 51:672-677 (2010)

Hiatt, S.M., Duren, H.M. Shyu, Y., Ellis, R.E., Hisamoto, N., Matsumoto, K., Kariya, K., Kerppola, T.K., and Hu, C.D.* *C. elegans* FOS-1 and JUN-1 regulate *plc-1* expression to control ovulation. *Mol. Biol. Cell* 20:3888-3895 (2009)

Xu, Y., Yang, W.H., Gerin, I., Hu, C.D., Hammer, G.D., and Koenig, R.J. DAX-1 and steroid receptor RNA activator (SRA) function as transcriptional coactivators for steroidogenic factor-1 in steroidogenesis. *Mol. Cell. Biol.* 29:1719-1734 (2009)

Yuan, Z., Gong, S., Song, B., Mei, Y., Hu, C., Li, D., Thiel, G., Hu, C.D., and Li, M. Opposing role for ATF2 and c-Fos in c-Jun-mediated apoptosis induced by potassium deprivation in cerebellar granule neurons. *Mol. Cell. Biol.* 29:2431-2442 (2009)

Deng, X., Liu, H., Huang, J., Cheng, L., Keller, E.T., Parsons, S.J., and Hu, C.D.* Ionizing radiation induces prostate cancer neuroendocrine differentiation through interplay of CREB and ATF2: Implications for disease progression. *Cancer Res.* 68:9663-9670 (2008)

Hiatt, S.M., Shyu, Y., Duren, H.M, and Hu, C.D.* Bimolecular fluorescence complementation (BiFC) analysis of protein interactions in living *C. elegans*. *Methods*, 45:185-191 (2008)

Vidi, P.A., Chemel, B.R., Hu, C.D., Watts, V.J. Ligand-Dependant Oligomerization of Dopamine D_2 and Adenosine A_{2A} Receptors in Living Neuronal Cells. *Mol. Pharmacol.* 74:544-551 (2008)

Shyu, Y., Suarez C.D., and Hu, C.D.* Visualizing ternary complexes in living cells using BiFC-FRET analysis. *Nat. Protocol.* 3:1693-1702 (2008).

Shyu, Y., Fox, SM., Duren, HM., Ellis, R.E., Kerppola, T.K. and Hu, C.D.* Visualization of protein interaction in living Caenorhabditis elegans using bimolecular fluorescence complementation (BiFC) analysis. *Nat Protocol*., 4:588-596 (2008).

Shyu, Y., Suarez, C., and Hu, C.D.* Visualization of AP-1-NF-κB ternary complexes in living cells by using a BiFC-based FRET. *Proc Natl Acad Sci U.S.A.*, 105:151-156 (2008).

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Liu, H., Deng, X., Shyu, Y., Li, J.J., Taparowsky, EJ., and Hu, C.D.***** Mutual regulation of c-Jun and ATF2 by transcriptional activation and subcellular localization. *EMBO J.*, 25:1058-1069 (2006).

Shyu, Y., Liu, H., Deng, X., and Hu, C.D.***** Identification of new fluorescent fragments for BiFC analysis under physiological conditions. *BioTechniques*, 40:61-66 (2006).

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Hu, C.D. and Kerppola, T. Simultaneous visualization of interactions between multiple proteins in living cells using multicolor bimolecular fluorescence complementation analysis. *Nat. Biotechnol*. 21, 539-545 (2003).

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Hu, C.D., Kariya, K., Okada, T., Qi, X., Song, C., and Kataoka, T. Effect of phosphorylation on activities of Rap1A to interact with Raf-1 and to suppress Ras-dependent Raf-1 activation , *J. Biol. Chem*., 274, 48-51 (1999).

Watari, Y., Kariya, K., Shibatohge, M., Liao, Y., Hu, C.D., Goshima, M., Tamada, M., Kikuchi, A., and Kataoka, T. Identification of Ce-AF-6, a novel *Caenorhabditis elegans* protein, as a putative Ras effector, *Gene*, 224, 53-58 (1998).

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Shirouzu, M., Morinaka, K., Koyama, S., Hu, C.D., Hori-Tamura, N., Okada, T., Kariya, K., Kataoka, T., Kikuchi, A, and Yokoyama, S. Interactions of the amino acid residue at position 31 of the c-Ha-Ras with Raf-1 and RalGDS, *J. Biol. Chem*., 273, 7737-7742 (1998).

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Yanagihara, C., Shinkai, M., Kariya, K., Yamawaki-Kataoka, Y., Hu, C.D., Masuda, T., and Kataoka, T. Association of elongation factor 1α and ribosomal

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Hu, C.D.* and Zhang, X.-H. Influence of EM on spleen cells NK activity and its mechanisms. *Chinese Journal of Microbiology and Immunol*ogy, 8, 11-14 (1989) (in Chinese).

Hu, C.D. * and Zhang, X.-H. Influence of EM on specific immune responses in normal Swiss mice. *Chinese Journal of Immunology*, 4, 176-178 (1988) (in Chinese).

b. Invited Peer-reviewed Review Articles

Hu, C.D. *, Choo, R., and Huang, J. Neuroendocrine differentiation in prostate cancer: a mechanism of radioresistance and treatment failure. *Front Oncol*, Apr 14;5:90. Doi: 10.3389/fonc.2015.00090 (2015)

Kodama, Y. and Hu, C.D.* Bimolecular fluorescence complementation (BiFC): A 5-year update and future perspectives. *Biotechniques*, 53:285-298 (2012)

Shyu, Y. and Hu, C.D.* Recent advances in fluorescence complementation-based technologies. *Trends Biotechnol.* 26:622-630 (2008)

Hu, C.D.*, Zhang, X.-H., and Bi, E.-H. Role of macrophages in the modulation of NK activity. *Foreign Medicine, Part of Immunology*, 10, 16-20 (1987) (*in Chinese*).

c. Invited Review Article (Not peer-reviewed)

Shyu, Y., Akasaka, K., and Hu, C.D*. Bimolecular fluorescence complementation (BiFC): A colorful future in drug discovery. Sterling-Hoffman Life Science Journal, July, 2007. (http://www.sterlinglifesciences.com/newsletter/articles/article006.html).

d. Book Chapters

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Ejendal, K.F.K., Conley, J.M., Hu, C.D. and Watts, V.J. Bimolecular fluorescence complementation analysis of G protein-coupled receptor dimerization in living cells. *Methods Enzymol*., 521:259-279 (2013).

Kodama, Y. and Hu, C.D.* Bimolecular fluorescence complementation (BiFC) analysis of protein-protein interaction: How to calculate signal-to-noise ratio. *Methods Cell Biol*., 113: 107-121 (2013).

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Hu, C.D., Grinberg, A.V. and Kerppola, T.K. Visualization of Protein Interactions in Living Cells Using Bimolecular Fluorescence Complementation (BiFC) Analysis. (ed. Coligan JE, Dunn BM, Speicher DW, Wingfield PT) *Curr. Protoc. Protein Sci.* 41:19.10.1-19.10.21. Hoboken, John Willey & Sons, 2005.

Hu, C.D. and Kerppola TK. Direct visualization of protein interactions in living cells using bimolecular fluorescence complementation analysis. *Protein-Protein Interactions* (ed. P. Adams and E. Golemis), Cold Spring Harbor Laboratory Press. Pp673-693, 2005.

Hu, C.D., Grinberg A., and Kerppola TK. Visualization of protein interaction in living cells using bimolecular fluorescence complementation (BiFC) analysis. In *Curr. Protoc. Cell Biol.* (ed. Bonifacino JS, Dasso M, Harford JB, Lippincott-Schwartz J, Yamada KM) pp. 21.3.1-21.3.21. Hoboken, John Willey & Sons, 2005

Kataoka, T., Kariya, K., Yamawaki-Kataoka, Y., Hu, C.D., Shirouzu, M., Yokoyama, S., Okada, T., and Shima, F. Isoprenylation-dependent and independent interaction of Ras with its effectors. In Kuzumaki, N. Cytoskeleton and G-Protein in the Regulation of Cancer. *Hokaido University Medical Library Series*, 37, 141-146 (1998).

Current and Past Grant Support at Purdue University as PI or Co-PI (2003-2017):

Active Grant Support

Title: Role and targeting of PRMT5 in prostate cancer
Source: NCI RO1
Role: Contact PI (**Multi-PI** with Chenglong Li and Jiaoti Huang)
Total Cost Requested: $2,590,428
Grant Period: 06/09/2017-05/31/2022
Goal: The goal of this proposal is to elucidate the molecular mechanisms by which PRMT5 promotes prostate cancer cell growth, improve the potency of BLL3.3, and conduct a preclinical evaluation of PRMT5 inhibition for castration resistant prostate cancer treatment.

Title: Co-targeting of androgen synthesis and androgen receptor expression as a novel treatment for castration resistant prostate cancer
Source: DoD (2015 PCRP)
Role: PI
Grant Period: 08/01/16-07/30/19
Total Cost: $557,000
Goal: The goal of this project is to evaluate whether co-targeting of androgen synthesis by abiraterone and androgen receptor expression via PRMT5 inhibition is an effective treatment for CRPC.

Title: Targeting neuroendocrine differentiation for prostate cancer radiosensitization
Source: DoD (2012 PCRP)
Grant Period: 09/30/13-09/30/17
Total Cost: $559,055
Role: PI
Goal: The goal of this grant is to use CREB targeting as a model to determine whether targeting radiation-induced NED can be explored as a novel radiosensitization approach for prostate cancer radiotherapy.

Title: Developing novel therapeutic strategies for castration-resistant prostate cancer
Source: DOoD (2013 PCRP)
Total Cost: $525,568
Role: Co-PI (PI: Kavita Shah)
Grant Period: 08/01/14-07/30/18 (no cost extension for current year)
Goal: The goal of this project is to determine whether targeting LIMK2 can be used to treat CRPC.

Title: Development of novel small molecule inhibitors targeting protein arginine methyltransferase 5
Source: CTSI (Indiana Drug Discovery Alliance)
Period: 12/01/14-12/30/17 (No cost extension for current year)
Total amount awarded: $10,000
Role: PI

Goal: The goal of this project is to discover inhibitors for disruption of PRMT5/MEP50 interaction using BiFC-based screening.

Title: Discovery of novel therapeutic targets for neuroendocrine prostate cancer
Source: Department of MCMP Research Enhancement Award, Purdue University
Period: 04/01/17-03/30/18
Total amount awarded: $50,000
Role: PI
Goal: The goal of this award is to discovery altered ion channels in neuroendocrine prostate cancer as therapeutic targets

Past Grant Support at Purdue University (2003-2016):

External Funding

Title: Temporal and spatial interaction patterns of bZIP proteins in living *C. elegans*
Source: National Science Foundation (MCB 0420634)
Role: PI
Grant Period: 08/15/04 – 07/30/08
Total Cost: $ 458,000
Goals: The goal of this project was to establish *C. elegans* BiFC assay to visualize temporal and spatial interactions of *C. elegans* bZIP proteins.

Title: Temporal and spatial interaction patterns of bZIP proteins in living *C. elegans*
Source: National Science Foundation (MCB 0420634)
Role: PI
Grant Period: 06/04/07 – 07/30/08
Total Cost: $4,750
Goals: The goal of this REU was to support Summer High School Student Research on the funded NSF *C. elegans* project.

Title: Regulation of *c-jun* transcription by ATF2 in cardiomyocyte in response to stress
Source: American Heart Association (AHA 0655570Z)
Role: PI
Grant Period: 07/01/06 – 06/30/08
Total Cost: $132,000
Goals: The goal of this project was to study the role of ATF2 subcellular localization in regulating *c-jun* transcription in rat cardiomyocytes in response to hypoxia and oxidative stress.

Title: Interplay of CREB and ATF2 in radiation-induced prostate cancer transdifferentiation

Source: DoD Prostate Cancer Idea Development Award (PC073981)
Role: PI
Grant Period: 06/01/08-05/30/11
Total Cost: $571,875
Goals: The goal of this project was to determine how CREB and ATF2 oppose each other at the transcriptional level to regulate radiation-induced neuroendocrine differentiation in prostate cancer cells.

Title: Improvement of BiFC technology and its application in the TLR signal transduction pathway (International collaborative project)
Source: Natural Science Foundation of China
Role: PI
Grant Period: 01/01/11-12/31/13
Total Cost: $35,000
Goal: The goal of this project was to collaborate with Dr. Yayi Hou at Nanjing University to apply BiFC technologies to study the TLR signaling in immune system.

Title: D2 receptor-induced sensitization of adenylate cyclase
Source: NIH RO1 (National Institute of Mental Health)
Role: Co-Investigator (PI: Val Watts)
Grant Period: 08/15/11-04/31/14
Total Cost: $770,922
Goal: The goal of this RO1 grant was to investigate the molecular mechanisms underlying D2 receptor-induced sensitization of adenylate cyclase. As a Co-Investigator, Dr. Hu provided his expertise in BiFC technology to help the analysis of D2 receptor interacting proteins.

Title: New mechanism for modulating opioid receptor mediated analgesia
Source: Showalter Trust Award
Role: Co-PI (PI: Richard van Rijn)
Total Cost: $75,000
Grant Period: 07/01/14-06/30/16
Goal: The goal of the project is to study the mechanisms and regulation of opioid receptors and to develop agents targeting protein-protein interactions using BiFC-based technologies.

Title: Targeting PRMT5 as a novel radiosensitization approach for primary and recurrent prostate cancer radiotherapy
Source: DoD (2011 PCRP)
Role: PI
Grant Period: 08/01/12-07/30/16
Total Cost: $559,269.91
Goal: The goal of this grant is to determine that PRMT5 is a novel therapeutic target for prostate cancer radiotherapy.

Title: Identification of the Ac5 sensitization interactome using BiFC

Source: NIH R21 (National Institute of Mental Health)
Role: Multi-PI with Val Watts
Total Cost: $463,111
Role: Multi-PI
Grant Period: 07/19/13-06/15/17
Goal: The goal of this project is to develop BiFC-based cDNA library screening for identification of Ac5 interacting proteins.

Internal Funding

Title: PRMT5 in prostate cancer development, progression and therapy response
Source: EVPRP Targeted RO1
Period: 12/01/15-05/30/17
Total amount awarded: $30,000
Role: PI
Goals: The goal of this project is to generate genetically modified mouse models (PRMT5 transgenic mice and PRMT5 Floxed mice) for prostate cancer research.

Title: Discovery of PRMT5 target genes in neuroendocrine prostate cancer
Source: Purdue University Center for Cancer Research
Period: 12/01/16-06/30/17
Total amount awarded: $10,000
Role: PI
Goals: The goal of this grant is to perform RNA-seq and ChIP-seq to identify target genes of PRMT5 contributing to the development of neuroendocrine prostate cancer.

Title: Mass spectrometric identification of pCREB interacting proteins in prostate cancer cells LNCaP
Source: Purdue Cancer Center Small Grant (Indiana Elks, Inc)
Role: PI
Grant Period: 03/01/08-02/28/09
Total Cost: $10,000
Goals: The goal of this project was to identify cytoplasmic interacting proteins of pCREB using mass spectrometry.

Title: Identification of interacting proteins and phosphorylation of ATF2 implicated in prostate cancer transdifferentiation
Source: Purdue Research Foundation
Role: PI
Grant Period: 06/01/08-05/30/09
Total Cost: $16,835
Goals: The goal of this PRF support was to use mass spectrometry to identify interacting proteins and phosphorylation of ATF2 in the cytoplasm in radiation-induced neuroendocrine cells and to determine how ATF2 nuclear import is impaired by ionizing

radiation.

Title: Targeting of prostate cancer transdifferentiation and proliferation via a novel DNA nanotube-based nucleic acid delivery
Source: Lilly Seed Grant
Role: PI
Grant Period: 01/01/09-12/31/10
Total cost: $100,000
Goal: The goal of this grant was to collaborate with Dr. Chengde Mao to develop DNA nanotube-based delivery of siRNAs.

Title: Targeting neuroendocrine differentiation as a novel therapeutics in prostate cancer treatment
Source: Purdue Research Foundation
Role: PI
Grant Period: 08/01/2010-07/30/2011
Total cost: $17,000
Goal: The goal of this project was to support graduate student Chris Suarez to study the role of radiation-induced neuroendocrine differentiation in radioresistance.

Title: Ionizing radiation induces neuroendocrine differentiation in nude mice prostate cancer xenograft models: Implication in disease progression
Source: Purdue University Center for Cancer Research
Role: PI
Grant Period: 01/01/09-12/31/11
Total Cost: $50,000
Goals: The goal of this project was to use xenograft nude mice prostate cancer cell models to investigate whether CREB and ATF2 contribute to radiation-induced neuroendocrine differentiation *in vivo* and to determine whether radiation induces changes of pCREB and ATF2 subcellular localization.

Title: Generation of cytoplasmic-localized ATF2 transgenic mice for prostate cancer research
Source: Purdue University Center for Cancer Research
Role: PI
Grant Period: 06/01/10-05/30/11
Total cost: $2,000
Goal: The goal of this support was to supplement the cost for making a transgenic mouse strain using the shared transgenic mouse facility

Title: Chromogranin A, a novel biomarker to monitor radiation-induced neuroendocrine differentiation in prostate cancer patients
Source: The Indiana Clinical and Translational Science Institute (CTSI)-Purdue Project Development Program
Role: PI
Grant Period: 06/01/10-05/30/12

Total cost: $10,000
Goal: The goal of this support was to conduct a pilot clinical study to determine the effect of radiotherapy on neuroendocrine differentiation in prostate cancer patients.

Title: Acquisition of an Nikon A1 Confocal Microscope
Source: Lilly Seed Grant, College of Pharmacy
Role: PI
Grant Period: 07/01/11-06/30/12
Total amount awarded: $300,000
Goal: The goal of this support was to acquire Nikon A1 confocal microscope to set up a Pharmacy Live Cell Imaging Facility

Title: Ultrahigh performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry
Source: Office of the Vice President for Research (OVPR) Laboratory Equipment Program
Role: Co-PI (PI: Andy Tao)
Period: Purchased by May 31, 2014
Total amount awarded: $100,000
Goal: The goal of this internal support was to acquire UHPLC.

Title: Generation of PRMT5 transgenic mice for prostate cancer research
Source: Purdue University Center for Cancer Research Shared Resource Grant
Period: 12/01/15-12/31/16
Total amount awarded: $3,100
Role: PI
Goal: The goal of this project is to use the transgenic mouse facility to generate PRMT5-overexpressing mice.

Past Grant Support at Kobe University as PI (1998-2001): $80,000

Title: Regulation of Rap1A activity by phosphorylation
Source: Kobe University, President Young Investigator Award
Role: PI
Grant Period: 04/01/98-03/30/99
Total Cost: ~$10,000 (for supplies)
Goals: The goal of this project was to investigate whether phosphorylation of Rap1A by PKA affects the ability of Rap1A to antagonize the function of Ras in activating Raf-1.

Title: Effect of phosporylation on the regulation of Rap1A activity
Source: Ministry of Education, Science, Sports, and Culture of Japan
Role: PI
Grant Period: 04/1/98 - 03/30/99
Total Cost: ~$ 10,000 (for supplies)

Goals: The goal of this project was to investigate whether phosphorylation of Rap1A by PKA affects the ability of Rap1A to activate downstream effectors such as Raf-1 and B-Raf.

Title: Activation mechanism of phospholipase C (PLC-ε) by Ras
Source: Hyogo Science and Technology Association
Role: PI
Grant Period: 04/01/00 – 03/30/01
Total Cost: ~$ 30,000 (for supplies)
Goals: The goal of this project was to investigate whether Ras regulates catalytic activity of PLC ε directly by their physical interaction. The approach was to use *in vitro* reconstitution system.

Title: Regulation of a novel phospholipase C (PLC-ε) by Ras
Source: Japan Society for the Promotion of Science
Role: PI
Grant Period: 04/01/00 – 03/30/01
Total Cost: ~$ 30,000 (for supplies)
Goals: The goal of this project was to investigate how Ras regulates catalytic activity of PLC ε and determine whether membrane anchoring of PLC-ε by Ras is sufficient for the activation of PLC-ε. This project was primarily focused on the studies in cells.

Note: Research grants in Japan do not provide personnel support. All faculty members and staff are supported by the government. Postdoctoral fellows and graduate students can only be supported by fellowships.

Fellowships/Awards received by trainees

- Susan Fox, Ross Fellowship (08/2003-07/2005): ~$56,000
- Susan Fox, 2nd place of graduate student presentation
  2004 Walther Cancer Institute Annual Retreat (Aug. 5-7)
- John Y Shyu, graduate student, Travel Award from 15th International Worm
  Meeting (June 25-29, 2005, Los Angeles) ($866)
- Susan Fox, graduate student, Travel Award from 15th International Worm Meeting (June 25-29, 2005, Los Angeles) ($866)
- Zeina Shtaih, Pharmacy Student, Summer Research Fellowship (2005 Breast Cancer Research Program), $4,000
- Jonathan Smith, Pharmacy Student, Summer Research Fellowship (2005 Breast Cancer Research Program), $2,000
- Jonathan Smith, NSF, Summer Research Fellowship (REU), $6,000 (IC $1,000)
- Apinya Supatkul, Prepharmacy Student, 2006 Summer Research Fellowship ($3,000)

- John Shyu, 1st Place of 2007 Purdue University Graduate Student Research Competition ($500)
- Holli Duren, Travel Award from 16th International Worm Meeting (June 27-July 1, 2007, UCLA) ($300)
- John Shyu, John Koo Travel Award for Fall 2007 ($1,000)
- Holli Duren, Kienly Award for outstanding graduate student teaching assistant 2007, MCMP ($750)
- Holli Duren, 2007 PRF Summer Fellowship ($2,472.09)
- Holli Duren, 2008-2009 PRF Fellowship ($16,835)
- Chris Suarez, Purdue University Doctoral Fellowship (08/2007-07/2009): ~$56,000
- Susan Fox, Bilsland Dissertation Fellowship (07/2008-12/2008): ~$14,000
- John Shyu, Bilsland Dissertation Fellowship (07/2008-12/2008): ~$14,000
- Holli Duren, 2008-2009 Graduate Student Award for Outstanding Teaching at Purdue University
- Holli Duren, 2009 Charles J. Paget Travel Award: $1,000
- Yutaka Kodama, 04/01/09-03/31/10 TOYOBO Postdoctoral Fellowship (~$34,000)
- Akhil Shenoy (Texas AM U) , 06/01/09-07/26/09, Purdue SROP: $5,000
- Yutaka Kodama, 04/01/10-03/31/12, JSPS Postdoctoral Fellowship (~$80,000)
- Holli Duren, Bilsland Dissertation Fellowship (01/01/2010-06/30/2010): $14,000
- Chih-chao Hsu, Ronald W. Dollens Graduate Scholarship in Life Sciences (08/2010-05/2011): $5,000
- Yeo Jin Choi, Purdue University College of Pharmacy 2010 Summer Undergraduate Research Fellowship: $3,000
- Chris Suarez, 2010 PRF Fellowship: $17,000
- Chih-chao Hsu, Travel Award for conference attendance from PULSe, $250 (2012)
- Chih-chso Hsu, 2011 PRF Fellowship: $17,000
- Chris Suarez, 2011 Paget Travel Award from MCMP department, $1,000
- Chris Suarez, 2012 AACR Minority Scholar in Cancer Research Award for participation in the Advances in Prostate Cancer Research conference (Feb 6-9, 2012), $1,800
- Chih-chao Hsu, Bilsland Dissertation Fellowship (09/01/12-12/31/12): $14,000
- Huantin Zhang (visiting student from Jinan University, China): Graduate Student Study Abroad Scholarship: $9,000 (2012)
- Huantin Zhang (visiting student form Jinan University, China): China Scholarship Council (CSC): $33,600 (awarded for two years 10/2013-9/2015, but stay for one year)
- Limin Zhang (PharmD student): 2014 Summer Undergraduate Research Fellowship (Lilly Endowment Fellowship): $4,800

- Jake Owens, Ross Graduate Fellowship (2014-2015), $38,000
- Athena He: 2016 LSAMP Summer Undergraduate Research Fellowship: $4,800
- Jonathan Malola: 2017 College of Pharmacy Summer Undergraduate Research Fellowship: $4,800
- Jake Owens, CTSI Predoctoral fellowship (07/01/17-06/30/19): $24,500/year plus tuition remission
- Jake Owens, 2nd place of Presentation Award at the 2017 Indiana Urological Research Symposium: $500

Teaching Experience

Lectures and labs

5/1985-6/1987: Microbiology and Immunology labs (medical students)

7/1987-8/1991: Epidemiology lectures and labs in the Department of Epidemiology, School of Public Health, Tongji Medical University, Wuhan

4/1997-8/2000: Physiology and Molecular Biology lab (medical students) in the Department of Physiology II, Kobe University

8/2003-present: As a faculty member in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University College of Pharmacy, I have been involved in the teaching of the following courses. The class size for the courses ranges from 5~15 for graduate students, 30-40 for BSPS students, and 150 ~205 for professional pharmacy students. The total number of lecture hours taught is approximately 40h/year. Teaching evaluation scores have been 4.5~4.8/5.0. In April 2017, I received the first teaching award of the Pharmaceutical Sciences Teacher of the Year, which was completely nominated and voted by BSPS graduates in the College of Pharmacy.

Courses Taught

Professional Pharmacy Students:
MCMP 305 (Biochemistry I, 2004-2006)
MCMP 304 (Biochemistry II, 2005-2008)
MCMP 440 (Pathophysiology, 2006-2012)
PHRM 824 (Principles of Pathophysiology and Drug Action, 2012-present)
PHRM 302 (Integrated Lab, Neoplasia module, 2005-2012)
PHRM 820 (Professional Program Laboratory, Neoplasia module, 2012-2015)

Graduate students:

MCMP 618/690G (Molecular Targets of Cancer, 2007-present)
MCMP 617/690N (Molecular Targets of Neurological Disorders, 2007-present)
MCMP 514 (Biomolecular Interactions-Theory and Practice, 2009-present)
MCMP 696 (Seminars in Medicinal Chemistry and Molecular Pharmacology, 2006-2008)
MCMP 599 (Cumulative written examinations, 2015-present)

Undergraduate students (BS in Pharmaceutic Sciences):
PHRM 460 (Drug Discovery and Development I, 2013-present)

MCMP 544 (Drug Classes and Mechanisms, 2015-present)

Medical students (Indiana School of Medicine):
LCME 504 (Molecular Cell Biology, guest lecture of Molecular Biology of Cancer, 2013-2015)

Courses Served as Coordinator

PHRM 824 (Principles of Pathophysiology and Drug Action, 2013-present)
MCMP 440 (Pathophysiology, 2011-2012)
MCMP 696 (Seminars in Medicinal Chemistry and Molecular Pharmacology, 2006-2008)
MCMP 599 (Cumulative written examinations, 2015-present)

Supervision of graduate, professional and undergraduate student research

07/1987-08/1991	Supervised 6 undergraduate students at Tongji Medical University
04/1997-08/2000	Co-supervised 7 Ph.D. students for thesis research with Professor Tohru Kataoka and supervised 5 undergraduate summer research at Kobe University.
09/2000-06/2003	Supervised two undergraduate students at University of Michigan
07/2003-present	(1) Served as thesis adviser of 8 Ph.D. students (6 graduated) and 2 master students (graduated) and co-adviser of 5 Ph.D. students (4 graduated) (2) Served as a thesis committee member of 45 graduate students (3) Served as a committee member of 36 oral preliminary examination (4) Supervised 39 graduate students for lab rotations (5) Supervised 31 professional and undergraduate student research (6) Supervised 4 high school students for summer research

Supervision of postdoctoral fellows, visiting scholars and technicians

07/2003-present	Supervised 9 postdoctoral fellows, visiting scholars and technicians

Service Experience

Major Administrative Services in the Purdue University Center for Cancer Research

2010-2013	**Seminar Coordinator** of Purdue University Center for Cancer Research
2010-Present	**Co-leader** of Prostate Cancer Discovery Group of Purdue University Center for Cancer Research
2012- Present	**Coordinator** of Indian Basic Urological Research (IBUR) monthly meetings
2012- Present	**Executive Committee Member** of Obesity and Cancer Discovery Group, Purdue University Center for Cancer Research
2013- Present	**Executive Member** of Purdue University Center for Cancer Research
2013- Present	**Co-leader**, Cell Identity and Signaling (CIS) Program of Purdue University Center for Cancer Research

Major Administrative Services at Purdue University

2007-2009	PULSe Graduate Program Admission Committee
2007-2009	PULSe Graduate Program Recruitment Committee
2008-present	Bindley Imaging Committee (BIG)
2010	Faculty Search Committee for a Cancer biology and Pharmacology position in the College of Veterinary Medicine
2012-present	PULSe Graduate Program Curriculum Committee

Major Administrative Services in the College of Pharmacy

2009-2013	Assessment Committee
2011-present	Director of Pharmacy Live Cell Imaging Facility (PLCIF)
2011-present	Chair of PLCIF Committee
2012-2014	Grade Appeal Committee
2012-present	Faculty Liaison for Core-Pharmacy Courses Taught by Other Schools (BIOL110/111)
2013-2014	Honor Degree Policy Committee
2013-2016	Curriculum committee
2014-present	Pharm.D. Academic Standards and Readmissions Committee

Major Administrative Services in the Department of Medicinal Chemistry and Molecular Pharmacology

2005-2011	Facility and Instrumentation Committee
2008-2009	Strategy Plan Task Force
2009	Biochemistry Task Force

2010	Business Manger Search Committee
2011	Faculty Search Committee (Pharmacology)
2012	Faculty Search Committee (Pharmacology)
2012	Faculty Search Committee (Epigenetics)
2010-2015	Graduate Admissions and Recruiting Committee
2012-present	Graduate Assessment Committee
2015-present	Chair of Graduate Assessment Committee
2016	Chair of faculty search committee